The Initiation of Adenovirus DNA Replication

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The Initiation of Adenovirus DNA Replication

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Had I the heavens' embroidered cloths,
Enwrought with golden and silver light,
The blue and the dim and the dark cloths
Of night and light and the half-light,
I would spread the cloths under your feet:
But I, being poor, have only my dreams;
I have spread my dreams under your feet;
Tread softly because you tread on my dreams.

W. B. Yeats.

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TABLE OF CONTENTS

		Page
1.	INTRODUCTION.	1
1.1.	A brief survey of a model system	1
1.2.	Structure of adenovirus	2
1.2.1.	The inverted terminal repetition (ITR)	5
1.2.2.	The terminal protein (TP)	6
1.3.	Replication of adenovirus DNA	8
1.4.	Initiation of adenovirus replication - a novel mechanism	11
1.5.	Genomes with covalently-linked proteins	15
1.6.	Mouse adenovirus FL	16
2.	MATERIALS.	19
2.1.	Abbreviations	19
2.2.	Cells	20
2.3.	Virus	20
2.4.	Sera	20
2.5.	Bacteria	21
2.6.	Chemicals and other substances	21
2.7.	Buffers and other solutions	23
3.	METHODS.	25
3.1.	Cell culture	25
3.1.1.	HeLa cells	25
3.1.2.	A549 cells	25
3.1.3	3T3 cells	25
3.2.	Mycoplasma test	26
3.3.	Growth of adenovirus	26
3.3.1.	Ad2 on HeLa cell monolayers	26
3.3.2.	Ad2 in HeLa cell suspension cultures	27

3.3.3.	AdFL on 3T3 cell monolayers	28
3.4.	Determination of virus titres	28
3.4.1.	Plaque tests with Ad2	28
3.4.2.	Immunofluorescence with AdFL	29
3.5.	Isolation of adenovirus DNA-protein complex	30
3.6.	Preparation of adenovirus DNA	31
3.6.1.	Digestion of DNA with restriction enzymes	31
3.7.	Preparation of nuclei from adenovirus infected cells	32
3.7.1.	Ad2 infected HeLa cells	32
3.7.2.	AdFL infected 3T3 cells	33
3.7.3.	AdFL infected HeLa cells	33
3.8.	Preparation of extracts	33
3.8.1.	Nuclear extracts	33
3.8.2.	Cytoplasmic extracts	34
3.9.	Gel electrophoresis	34
3.9.1.	PAA gels	34
3.9.2.	Agarose gels	35
3.10.	Autoradiography	35
3.11.	*In vitro* formation of the initiation complex	36
3.12.	Growth of bacteria	37
3.12.1.	Isolation of plasmid DNA	37
4.	RESULTS.	39
4.1.	Introduction	39
4.1.1.	Replication of adenovirus *in vitro*	40
4.2.	Preparation of nuclear extracts from adenovirus infected cells	41
4.2.1.	Influence of hydroxyurea on the *in vitro* activity of nuclear extracts	42
4.2.2.	Variation of dCTP concentration in the *in vitro* assay	44
4.2.3.	Omission of ATP in the initiation reaction	44
4.2.4.	Omission of ddATP in the initiation reaction	44
4.2.5.	Preincubation of nuclear extracts	45

4.2.6.	Aphidicolin in the *in vitro* assay	46
4.2.7.	The *in vitro* initiation assay	47
4.3.	Initiation of adenovirus replication *in vitro*	48
4.3.1.	Initiation and limited elongation of Ad2 DNA *in vitro*	48
4.3.2.	DNA-protein complex is required for the initiation reaction	50
4.3.3.	Stability of the DNA-protein complex	50
4.3.4.	Initiation and limited elongation of AdFL DNA *in vitro*	52
4.3.5.	DNA-protein complex is required for the AdFL initiation reaction	54
4.3.6.	Initiation on DNA-protein complex templates occurs only in homologous *in vitro* systems	55
4.3.7.	Nuclear extracts from "mixed" adenovirus/host cell systems retain their template specificity	56
4.3.8.	Homologous versus heterologous systems using natural templates	58
4.3.9.	Estimation of the molecular weigth of the adenovirus precursor terminal protein	59
4.4.	Role of nuclear and cytoplasmic factors in adenovirus DNA replication	61
4.4.1.	Host cell nuclear factors are necessary for efficient *in vitro* initiation of Ad2 DNA replication	61
4.5.	Initiation of adenovirus replication on protein-free DNA templates	65
4.5.1.	Plasmids containing adenovirus terminal sequences	66
4.5.2.	Cloned termini of Ad2 DNA support the formation of the initiation complex	70
4.5.3.	Cloned termini of AdFL DNA also support formation of the initiation complex *in vitro*	72
4.5.4.	Cloned termini of AdFL DNA support complex formation using "mixed" extracts from adenovirus infected cells	74

5.	DISCUSSION.	78
5.1.	The structure of a functional template	78
5.2.	Host cell components in adenovirus DNA replication	91
6.	SUMMARY.	94
6.1.	ZUSAMMENFASSUNG.	96
7.	LITERATURE.	98

1. INTRODUCTION .

1.1. A brief survey of a model system.

The exciting developments in adenovirus biology over the last ten years have made this animal virus one of the most talked-about DNA molecules among virologists today. Its success as a model system for the study of eukaryotic DNA replication is based on several features of the viral structure and mode of propagation in infected host cells.

A prerequisite to an understanding of the complex events which occur during the replication of eukaryotic chromosomes is a simplified system in which a nucleic acid molecule is faithfully replicated by a defined number of proteins, at least some of which should be of cellular origin. If this simplified system may be reproduced *in vitro*, it is then possible, using modern biochemical techniques, to purify and characterize the components of the model replication system. Such a procedure has been adopted in the study of prokaryotic DNA replication where bacteriophages and plasmids have served successfully to elucidate much of the basic information about the synthesis of DNA in bacteria. Adenovirus infected human cells can be grown in very large quantities in culture. The efficiency of infection is high (about 5×10^5 virus particles per cell) and thus, large amounts of virus may be easily purified for further analysis. There are many DNA-negative temperature-sensitive (ts) mutants of Ad2, Ad5 and Ad12 available which are very useful in understanding DNA replication and in particular, for localizing gene functions on the linear genome. The structure and genetic organization of the adenovirus genome are well defined and the complete primary sequence of Ad2 is now known.

Unlike the papovaviruses, adenovirus infection does not stimulate but rather turns off cellular DNA replication. Thus, at a late stage in infection (e.g. 18 hpi), host cell DNA synthesis is blocked permitting the unambiguous *in vivo* analysis of viral DNA replication. A corollary to this observation is that by inhibiting

total DNA synthesis (e.g. treatment with hydroxyurea), nuclear extracts may be prepared from infected cells which are capable of efficiently replicating adenovirus DNA *in vitro*. The development of this *in vitro* system was a major breakthrough in adenovirus biology since it allows an analysis of virus- and cell-coded proteins involved in DNA replication as well as the identification of adenovirus template requirements. With the exception of certain virus-coded proteins, adenovirus DNA replication involves cellular enzymes and other proteins and thus, the study of adenovirus DNA replication may illuminate cellular mechanisms of DNA synthesis. Finally, adenovirus serves not only as a model system for eukaryotic DNA replication but also contributes to the study of gene organization, gene expression and cellular transformation in mammalian cells. Adenovirus genes are expressed in an ordered fashion which is tightly regulated. The viral genome does not specify its own RNA polymerase and thus transcription by cellular enzymes probably occurs by mechanisms which operate in normal uninfected cells. Post-transcriptional processing is also evident and the splicing phenomenon was, for instance, first observed in the adenovirus system. With respect to transformation processes, it is known that all human adenoviruses can transform rodent cells in culture. Several studies, in particular, with adenovirus type 12, have implicated the left end of the viral genome as the "transforming" region and thus provided the means to investigate many aspects of cellular transformation.

1.2. Structure of adenovirus.

The adenoviruses are a group of DNA viruses associated with the respiratory tract of man and other animals. The first strains were isolated about thirty years ago (Rowe et al., 1953; Hilleman and Werner, 1954) and since that time, almost one hundred different serotypes have been characterized from a wide variety of animal species including the chicken, the tree shrew and man. The human adenoviruses, represented at present by 38 serotypes, have been subdivided into five groups (A-E) on the basis of DNA homology

(Green et al., 1979b). Two members of group C, adenovirus serotype 2 (Ad2) and serotype 5 (Ad5), have been studied intensively both *in vivo* and *in vitro*. Since they fail to produce tumours in newborn hamsters, the group C adenoviruses have been termed non-oncogenic; however, both Ad2 and Ad5 can cause phenotypic transformation of rodent cells in culture and these transformed cells can, in turn, lead to tumour formation when implanted into appropriate animals (Graham et al., 1974).

Adenoviruses are non-enveloped viruses of icosahedral structure. The viral particles consist of an outer coat or capsid, containing the major structural polypeptides, and a dense central core comprising one linear double-stranded DNA molecule associated with at least four core proteins (Philipson and Lindberg, 1974; Winnacker,1978). The linear uninterrupted DNA genome has a molecular weight of 20 - 25×10^6 daltons (Green et al., 1967) and theoretically, could code for 30-40 polypeptides of average size. For mapping purposes, it is necessary to distinguish between the right and left ends of a linear molecule and because the A/T-rich half of Ad2 DNA was first described as the right half (Doerfler and Kleinschmidt, 1970), this convention has now been adopted for all adenoviruses. Further, the two complementary DNA strands have been designated right (r) and left (l), indicating rightward and leftward transcription, respectively (Sharp, 1977).

Adenovirus DNA molecules have been shown to lack terminal redundancies, cohesive ends and circular permutations (Green et al., 1967). Thus, the absence of known structural features, associated with the replication of well-studied bacteriophage DNA molecules, presented problems in understanding the replication of the linear adenovirus genome. In particular, the mechanisms of initiation and termination of DNA synthesis remained completely obscure for many years. Within the last decade, two novel and unique features of the adenovirus genome were discovered; the inverted terminal repetition (ITR) and a protein linked to the termini of the DNA molecule, the terminal protein (TP).

```
AD2   CATCATC-ATAATATACCTTATT--TTGGATT-GAAGCCAATATGATAATGAGGGGGTGGAGTTTGTGACGTGGCGCGGGGCGTGG
AD5   CATCATCAATAATATACCTTATT--TTGGATT-GAAGCCAATATGATAATGAGGGGGTGGAGTTTGTGACGTGGCGCGGGGCGTGG
AD3   CTATCTATATAATATACCTTATAG-ATGGAAT-GGTGCCAACATGTAAAATGAGGTAATTTAAAAAAGTGCGCGCTGTGTGGTGATT
AD7   CTCTCTATATAATATACCTTATAG-ATGGAAT-GGTGCCAACATGTAAATGAGGTAATTTAAAAAAGTGCGCGCTGTGTGGTGATT
AD4   CTCTCTCTATAATATACCTTATTTTTTTTGTG-TGAGTTAATATGGCAATAAGGCGTGAAAATTTGGGGATGGGGCGCGCTGATTG
AD9   CTATCTATATAATATACCCCACAAAGTAAACA-AAAGTTAATATGCAAATGAGCTTTTGAATTTTAACGGTTTCGGGGCGGAGCCA
AD10  CTTCATCAATAATATACCCCACAAAGTAAACA-AAAGTTAATATGCAAATGAGCTTTTGAATTTTAACGGTTTCGGGGCGGAGCCA
AD12  CATCATCAATAATATACCTTATA--CTGGACT-AGTGCCAATATTAAAATGAAGTGGGCGTAGTGTGTAATTTGATTGGGTGGAGG
AD18  CATCATCAATAATATACCTTATA--CTGGACT-AGAGCCAATATTAAAATGAAGTGGGTGTGGCGATGTACTTTGATTGGGTGGAG
AD31  CATCATCAATAATATACCTTACA--CTGGACT-TGAGCCAATATTAAAATGAAGTGGGCGGAGTGAATAGTTAATTGACCGTAGGC
SA7   CATCATCAATAATATACCTTATT--TTGGGAACGGTGCCAATATGCTAATGAGGTGGGCGGAGTTTGGTGACGTATGCGGAAATGG
TAV   CATCATCAATAATATACCTGACACTTTTGACGT----------AATGACGGTTGCAAGTGCCACGTCGTCGTGGGCGTGTCT
ADFL  CATCATCAATAATATAC---A----------GTTAGCA-AAAAATGGCGCCTTTGTTTGGCTTTGTTCCAACTGTTTTTGG
CELO  GATGATGTATAATA-ACCTCAAAA----------ACTAACGCAGTCATAACCGGCCATAACCGCAGCGTGTCGC - 63
```

```
AD2   GAACGGGGCGGGTGACGTAG - 102
AD5   GAACGGGGCGGGTGACGTAG - 103
AD3   GGCTGCGGGGTTAACGGCTAAAAGGGGCGGCGCGACCGTGGGAAAATGACGT - 136
AD7   GGCTGTGGGGTGAATGACTAACATGGGCGGGGCGGCCGTGGGAAAATGACGT - 136
AD4   GCTGTGACAGCGGCGTTCGTTAGGGGCGGGG - 116 - CAGGTGACGTTTTGAT / TAACTGATGTGTTTAA
AD9   ACGCTGATTGGACGAGAGAAGACGATGCAAATGACGTCACGACGCACGGCTAACGGTCGCCGCGGAGGCGGGGC - 158
AD10  ACGCTGATTGGACGAGAGAAGACGATGCAAATGACGTCACGACGCACGGC - 135
AD12  TGTGGCTTTGGCGTGCTTGTAAGTTTGGGCGGATGAGGAAGTGGGGCGCGGCGTGGGAGCCGGCGCGCCGGTGTGACGT - 162
AD18  GTGTGGCCTGGGCGTGTTTGTAAGTTTGGGCGGATGAGGAAGTTGGGCGCGGCGTGGGAGCCGGCGCGCCGGTGTGACGTGT - 165
AD31  GTGGTTTGCAAGTTTGCCGAAGCCGGATGTGACGCGTGTGGGAGCCGGGCGCGCCGGATGTGACG - 148
SA7   GCGGAGTTAGGGGCGGGGTTTGGCGGTAGGCGTGGCT.....
TAV   TTTGTGACCTTTGGACGGGCGTTTCGCTGGCCGGGTTCCCAGTTTCGGGGCCGTTCCCGAGAACGTTGAGTCATGACAGCTGACCCGGG - 161
ADFL  CCCGAGTTGGGTTTCGTTTTCCCGGG - 93
```

Figure 1. DNA sequences of adenovirus inverted terminal repetitions.

The nucleotide sequences of known ITRs from several human and non-human adenoviruses are shown. Ad2 - Ad31 are human adenovirus serotypes; SA7 is the simian adenovirus; TAV is Tupaia (tree shrew) adenovirus; AdFL is a mouse serotype and CELO is the avian adenovirus. The numbers represent the length of the repeats in nucleotides. Conserved sequences in the A/T- and G/C-rich regions are indicated by boxes. Sequences which are also present at the origin of papovaviruses are underlined (waved line). Reproduced from Fütterer and Winnacker, 1984.

1.2.1. The inverted terminal repetition (ITR).

The presence of inverted terminal repetitions in the adenovirus genome means that the nucleotide sequence at one terminus of each strand of the DNA molecule is complementary to the nucleotide sequence at the other terminus of the same strand (Garon et al., 1972; Wolfson and Dressler, 1972). Denaturation and renaturation of intact DNA molecules thus generates single-stranded circles with a "panhandle" structure. The length of the inverted terminal repetition varies between 63 base pairs (CELO virus, chicken) and 165 base pairs (Ad12 and Ad18, human). The DNA sequences of the ITRs of many adenoviruses have been determined including several human serotypes, simian adenovirus SA7, mouse adenovirus FL, Tupaia (tree shrew) adenovirus and the avian adenovirus CELO (see fig. 1). So far, all ITRs extend to the very end of the viral genome; all sequences contain a 5'-terminal dC (the only known exception is CELO virus where the 5'-nucleotide is dG) and all display certain conserved features within the primary DNA sequence (see Stillman et al., 1982; Fütterer and Winnacker,1984).

The ITR may be divided into a 5'-proximal A/T-rich region of 50-52 base pairs and a distal G/C-rich region of 50-110 base pairs in length. Within this A/T-rich region a sequence of 10bp (ATAATATACC) is perfectly conserved in all human serotypes and somewhat less well conserved in other adenoviruses. The G/C-rich region, although

more variable, also contains two short sequence features that are common to most or all adenoviruses (fig. 1). The possible significance of these sequence features will be discussed later (see section 5.1).

1.2.2. The terminal protein (TP).

The association of protein with the termini of the adenovirus genome was originally demonstrated by Robinson et al.(1973) and Robinson and Bellet (1974). In the case of subgroup C of the human adenoviruses, a protein of M_r = 55000 daltons is covalently attached to each 5'-terminus of the linear double-stranded DNA molecule (Rekosh et al., 1977). The covalent nature of the linkage between the protein and the adenovirus genome was first suggested by treatment with various denaturing agents (Padmanabhan and Padmanabhan, 1977) and further supported by the insensitivity of the genome to the action of the 5'-specific exonuclease of the bacteriophage λ and to phosphorylation of the 5'-termini by sequential phosphatase and polynucleotide kinase treatment (Carusi, 1977). Over the last few years, the adenovirus terminal protein (TP) has been the subject of intensive study and today, many of the details concerning its structure and function are known.
The covalent linkage between adenovirus DNA and the terminal protein is a phosphodiester bond between the ß-hydroxyl group of a serine residue in the protein and the 5'-hydroxyl of the terminal deoxycytidine residue (Desiderio and Kelly, 1981). The terminal protein is derived from a larger precursor protein of M_r = 80000 daltons and this precursor terminal protein (pTP) is covalently attached to the termini of replicating DNA *in vivo* (Coombs et al., 1978; Kelly and Lechner, 1978; Stillman and Bellett, 1978, 1979; van Wielink et al., 1979; Challberg and Kelly, 1981) and to the 5'-end of nascent DNA synthesized *in vitro* (Challberg et al., 1980; Stillman, 1981). Partial proteolysis experiments have shown that the 80K protein (pTP) is closely related in structure to the mature 55K protein (TP) (Challberg et al., 1980). During the late phase of the infectious cycle, after assembly of the viral particle

(Fütterer, 1982), the 80K precursor is cleaved by a virus-encoded protease to its mature 55K form. The protease is coded by a late gene situated towards the right end of the genome. It is responsible for the cleavage of certain structural proteins (pVI, pVII and pVIII) to their mature virion forms (Weber, 1976; Mirza and Weber, 1977). A temperature sensitive mutant of Ad2, ts1, grown at the restrictive temperature (40°C) fails to cleave viral structural proteins during virus maturation (Bhatti and Weber, 1979). The genomes of these Ad2 ts1-40°C mutants contain DNA covalently linked to a terminal protein (80 - 87K) which is structurally related to the pTP discussed above (Challberg and Kelly, 1981; Stillman et al., 1981).The DNA is covalently linked to the protein, either the TP or pTP, by the same phosphodiester bond.

For many years, the origin of the terminal protein remained obscure and the possibility was raised that it may be of cellular origin (Green et al., 1979a). Recently, cell-free translation of virus-specific mRNA selected by hybridisation with the viral DNA l-strand resulted in the formation of an 87K protein (Stillman et al., 1981). This 87K protein has been identified as the precursor terminal protein (pTP) and is the same as the 80K protein described by Challberg et al. (1980); the discrepancy in size estimates is probably due to the use of different molecular weight markers. The messenger RNA for pTP was mapped between coordinates 11 and 31.5 on the viral genome and these mapping experiments led to the definition of a new early transcription unit, designated E2B. This new early region has a large coding capacity. Transcripts originate from a promoter at coordinate 75, include leaders at coordinates 75, 68.5 and 39 and these sequences are then spliced to three main bodies which extend from coordinates 30.3, 26.0 and 23.1 to terminate at coordinate 11.1.

A look at the DNA sequence between coordinates 0 and 32 reveals five open reading frames, two of which have remarkably large coding capacities (Alestrom et al., 1982; Gingeras et al., 1982). One of these starts with an ATG at nucleotide 10534 (28.9%) and ends with a TAG at nucleotide 8575 (23.4%) and, if translated, would encode a 74.6K polypeptide. The identity between this open reading frame and

pTP has been established based on a comparison of the methionine-containing tryptic peptides of pTP and those predicted from this sequence (Smart and Stillman, 1982). The mature 55K terminal protein is derived from the COOH-terminal of pTP and although the exact proteolytic cleavage site is at present unknown, a sequence around map coordinate 26.1 shows striking similarity to the cleavage site utilized for the maturation of protein pVI to the viral coat protein VI (Akusjärvi and Persson, 1981). The predicted value for the molecular weight of the pTP i.e. 74.6K, assuming that it is encoded exclusively within this open reading frame (28.9% - 23.4%), is significantly lower than the value estimated by SDS-polyacrylamide gel electrophoresis i.e. 80 - 87K. It is possible that a small amount of coding region from the leader at coordinate 39 is spliced to the main body of RNA and that this leader sequence might encode the NH_2-terminal portion of pTP. However, since the nature of the amino terminus of pTP remains unclear, a direct sequence analysis of the mRNA body-leader junction is clearly required to determine whether the open reading frame extends to the right of the sequence at coordinate 28.9.
In the past, much has been speculated on the role of the adenovirus terminal protein including possible functions in cellular transformation (Rekosh et al., 1977), regulation of transcription, viral encapsidation processes (Brown et al., 1975) and viral DNA replication. One known biological effect of the protein is that it increases the infectivity of adenovirus DNA by 3- to 100-fold as compared to protein-free DNA (Sharp et al., 1976; Chinnadurai et al., 1978; Estes, 1978). Recently, attention has focused on the role of the terminal protein in adenovirus DNA replication.

1.3. Replication of adenovirus DNA.
(Winnacker, 1978; Challberg and Kelly, 1982; Fütterer and Winnacker 1984).

The basic features of adenovirus DNA replication have been determined by several methods including electron microscopy, analysis of single-stranded DNA in replicating molecules and by

elucidation of the temporal order of synthesis of specific regions of the viral genome. Understanding the structure of the replication intermediates has led in turn to a reasonably clear picture of the mechanism involved in adenovirus DNA replication.
The model for Ad DNA replication is a two-step one and its features are summarized in figure 2. Replication can be initiated at either end of the double-stranded genome and synthesis of a daughter strand proceeds in the 5' to 3' direction with concomitant displacement of the parental strand of the same polarity. The intermediates in this step are full length linear duplices with at least one single-stranded branch (Type I molecules). The displaced parental strand then serves as template for DNA synthesis initiated at its 3' terminus and the result is the production of a second daughter molecule. The replication intermediates in the second step of the model are full-length unbranched DNA molecules with partial single- and double-stranded character (Type II molecules). There is also a low probability that some DNA molecules may be doubly initiated i.e. both parental strands may serve simultaneously as templates for daughter strand synthesis. In this case, two type II replication intermediates are produced when the oppositely moving displacement forks meet.

Important features of the present model for adenovirus DNA replication may be summarized as follows:

- the frequency of initiations at the two ends of the viral genome is approximately the same. Multiple initiation events on the replicating molecule are common but occur preferentially at the same end of the DNA. Thus, in general, growing points always move in the same direction (Revet and Benichou, 1981).
- all initiation events that occur during the replication of adenovirus DNA take place at the 3' terminus of the template DNA strand and all daughter strands grow from their 5' toward their 3' termini. Thus, DNA synthesis proceeds in a semi-conservative, asymmetric and continuous fashion and nascent daughter strands are elongated without the need for synthesis and subsequent joining of Okazaki fragments. A typical Cairns-type replication fork is

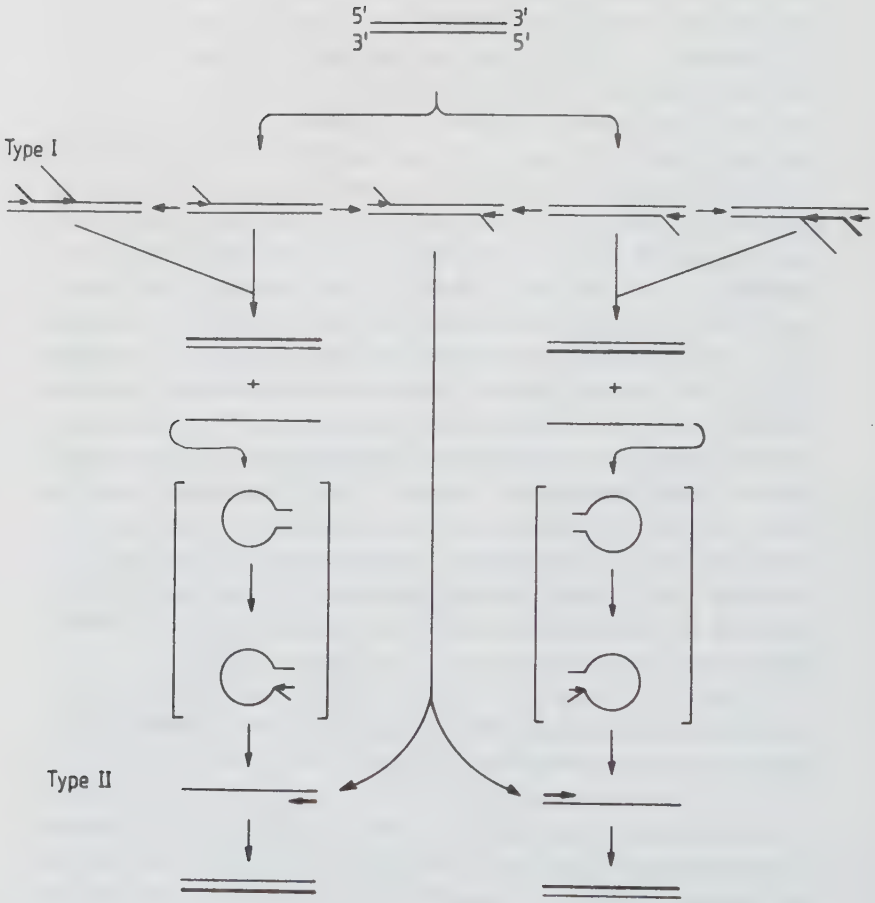


Figure 2. Model for adenovirus DNA replication.

The model shows the proposed mechanism of adenovirus DNA replication. Type I and type II refer to replicative intermediates of the viral DNA, while the single-stranded "panhandle" structures are purely hypothetical intermediate conformations. The model is described in detail in section 1.3. (Redrawn from Lechner and Kelly, 1977).

therefore not a feature of this model.

- since the nucleotide sequences at the 3' termini of the l- and r-strands of adenovirus DNA are identical, it seems probable that all initiation events proceed by the same molecular mechanism. The "panhandle" structures (Daniell, 1976) are hypothetical but possible replication intermediates and could be formed by intramolecular base-pairing between the termini of the displaced single-stranded DNA molecules. The duplex portions of r- and l-panhandles would be identical in structure with each other and indistinguishable from the molecular ends of the full-length duplex DNA genome. The structural equivalence of these template forms may be important for the recognition sites of enzymes involved in DNA replication. However, it must be emphasized that no panhandle structures have been found among replicating Ad DNA molecules even after *in vivo* crosslinking fixation. Therefore, the replication of the displaced parental strand is still an unsolved problem today and the possibility of two different initiation mechanisms for Ad DNA synthesis remains (Revet and Benichou, 1981).

1.4. Initiation of adenovirus replication - a novel mechanism.

The proposed mechanism of adenovirus DNA replication has been described in the preceding section. One of the most conspicuous features of this model is that initiation of replication occurs at either end of the double-stranded genome and proceeds in an asymmetric but continuous fashion from the 5'- towards the 3'-terminus. There is much experimental evidence to support the view that both origins and termini of viral DNA replication are located at the ends of the linear adenovirus genome. In particular, the asymmetric distribution of radioactive label along the genome and within the two DNA strands of restriction enzyme terminal fragments implicate the molecular ends of the genome as sites of initiation and termination of DNA synthesis (Schilling et al., 1975; Tolun and Pettersson, 1975; Bourgaux et al., 1976; Horwitz, 1976; Weingärtner et al., 1976; Ariga and Shimojo, 1977; Sussenbach and Kuijk, 1977; Ariga and Shimojo, 1978; Arens and Yamashita,

1978; Sussenbach and Kuikj, 1978a; Reiter et al., 1980). Since complementary strand synthesis apparently occurs without the need for short Okazaki fragments, the model assumes only one initiation event for the complete synthesis of each DNA strand. Further, the identity of the molecular ends of the genome (see 1.2.1. concerning the ITR) suggests that the same initiation event occurs at both the right and left ends of the linear molecule. Thus, the model for adenovirus DNA replication appears to be relatively simple.

However, the problem of linear replicons such as adenovirus, to initiate replication becomes evident when one considers the possible mechanisms of priming DNA synthesis. All known prokaryotic and eukaryotic DNA polymerases can initiate de novo DNA synthesis only in the presence of a free 3'-OH nucleotide (ribo- or deoxyribo-) terminus (Weissbach, 1975, 1977; Kornberg, 1980).The classical RNA priming scheme (Dressler, 1975) has the major disadvantage that after enzymatic removal of the oligoribonucleotide, the original problem of generating a DNA sequence at the 5'-terminus still remains. This problem is circumvented in some cases by temporarily converting a linear replicon into a circular or concatemeric molecule e.g. replication intermediates of the phages λ (early phase) and T7 (late phase) respectively (Tomizawa and Ogawa, 1968; Furth and Wickner, 1983; Watson, 1972). The formation of such intermediates is made possible by the presence of "sticky ends" (e.g. phage λ) or terminally redundant sequences (e.g. phage T7) at the ends of the linear genome. However, the presence of inverted terminal repetitions in the adenovirus genome does not allow formation of circles or concatemers from double-stranded DNA by the conventional mechanisms discussed above. The circular DNA molecules which, under certain conditions, may be isolated from virions (Robinson et al., 1973; Robinson and Bellett, 1974; Rekosh et al., 1977) and from adenovirus infected cell nuclei (Girard et al., 1977) are not covalently bound and probably arise via terminal protein interactions. These circular molecules are rapidly converted to linear double-stranded monomers upon treatment with proteases or with the detergent, SDS. A recent report of covalently closed circles of adenovirus DNA in infected permissive and nonpermissive

cells (Ruben et al., 1983) would suggest that such structures are possibly involved in integration but not in replication events.

A number of models involving "self-priming" has been put forward to explain the initiation of replication on the adenovirus linear genome. In particular, the proposal that foldback palindromic sequences at the 3'-termini might provide the necessary 3'-OH primer for DNA synthesis seemed to solve the problem of a free terminus (Cavalier-Smith, 1974). However, newly synthesized DNA has never been found covalently linked to parental DNA during adenovirus replication. Further, the necessary sequence arrangements required for the formation of such unusual "hairpin" structures are not present at the termini of the adenovirus genome (Stillman et al., 1977; Sussenbach and Kuijk, 1978b).

An alternative model for the initiation of adenovirus replication involving the DNA-protein complex has been put forward by Rekosh et al. (1977). This model is particularly interesting in that it obliterates the requirement for circular or concatemeric intermediates and for palindromic base sequences in the terminal regions of the DNA. Further, the model directly involves the precursor to the adenovirus terminal protein (pTP) which has been shown experimentally to be attached to replicating DNA *in vivo* and *in vitro* (see section 1.2.2.). The proposed model is shown diagrammatically in figure 3. According to the model, the primary initiation event is the formation of a covalent linkage between one molecule of the newly synthesized precursor terminal protein (pTP) and the 5'-terminal deoxynucleotide residue (dCTP in all human adenoviruses). This event is probably part of a concerted reaction in which the free pTP associates with either terminus of the parental DNA molecule. Correct alignment of this initiation complex (pTP-dCMP) at the extreme termini of the genome is believed to be mediated by interactions with the parental DNA terminal protein (TP) or with specific terminal nucleotide sequences or with both. The correctly- positioned initiation complex has a free 3'-OH group which may then serve as primer for subsequent chain elongation by DNA polymerase.

In contrast to the other proposed initiation models, the so-called "protein-priming" model discussed above has found much experimental

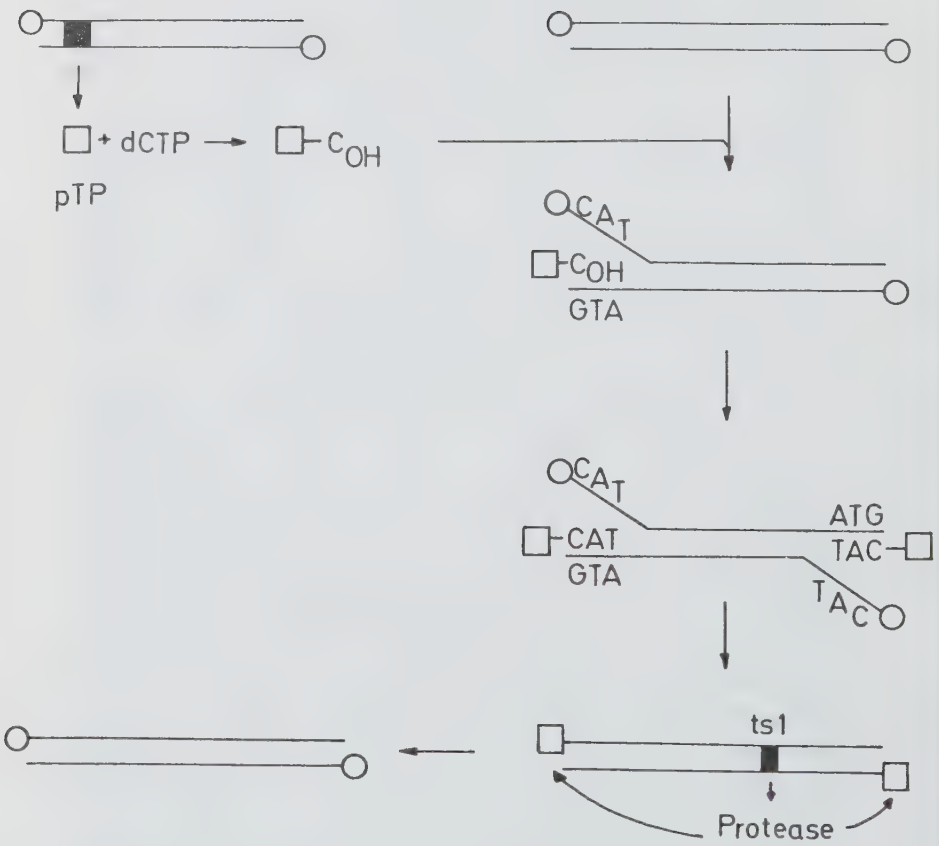


Figure 3. Model for the initiation of adenovirus DNA replication.

The virus-encoded precursor terminal protein (pTP) covalently binds dCTP to form the initiation complex. The pTP-dCMP complex may then serve as primer for subsequent DNA synthesis. During virus maturation, the pTP is processed by a protease, which is defective in the Ad2ts1 mutant, to produce the mature terminal protein on the DNA-protein complex.

□ =pTP; ○ =TP. (Modified from Stillman et al., 1981).

support. In particular, a recently developed *in vitro* system to monitor the initiation event (Lichy et al., 1981) has confirmed many of the basic elements of this model (see section 4.1.1.). Finally, it should be kept in mind that despite widespread support for the "protein-priming" model, there is no experimental evidence to date concerning the initiation of the displaced parental strand which may well involve a different molecular mechanism (see 1.3.).

1.5. Genomes with covalently-linked proteins.

The presence of protein covalently bound to DNA is not unique to the adenovirus family. The partially double-stranded circular genome of Hepatitis B virus (HBV) has a protein linked to or near the 5' end of its "complete" DNA strand (Gerlich and Robinson, 1980). Despite its small size and relatively simple structure, the parvovirus H1 has been shown to contain a protein (approx. 60000 - 70000 daltons in size) covalently linked to both 5'-termini of the double-stranded replicative form (RF) of its DNA (Revie et al., 1979). The role of these proteins in viral replication is at present unclear. DNA-protein complexes may also be formed transiently e.g. during the action of a topoisomerase on supercoiled DNA or during the replication of the duplex form of ØX174 DNA when the phage gene A product first nicks and then becomes covalently bound to the viral (+) strand of the RF DNA (Eisenberg et al., 1977; Eisenberg, 1980; Kornberg, 1980). The closest analogy with adenovirus however is, without doubt, the *Bacillus subtilis* phage Ø29. The DNA of phage Ø29 is a linear double-stranded molecule of about 18,000 base pairs in length and contains a viral protein, p3 (M_r = 27000 daltons), covalently bound to both 5'-termini (Harding et al., 1978; Ito, 1978; Salas et al., 1978; Yehle, 1978). The phage Ø29 terminal protein is an early phage product and has been shown to play a role in the initiation of phage DNA replication *in vivo* (Mellado et al., 1980) and *in vitro* (Penalva and Salas, 1982). Thus, the model for initiation of phage Ø29 DNA replication strongly resembles that for adenovirus DNA replication.

Finally, several single-stranded RNA viruses of both animal and plant origin are known to contain protein-linked genomes. Much is known about the protein bound genomes of picornaviruses (e.g. foot and mouth disease virus, Hepatitis A virus etc.) and in particular, about poliovirus. The RNA genome of poliovirus contains a 22-amino acid protein (VPg) linked to the 5'-terminal uridine residue (Lee et al., 1977; Kitamura et al., 1981). The VPg of poliovirus is believed to play a role in the initiation of RNA synthesis and in the recognition of virion RNA by procapsid precursors in viral morphogenesis (Nomoto et al., 1977).
Considering the many different examples of genome-linked proteins, the novel initiation mechanism involving "protein-priming" which is proposed for adenovirus DNA replication, may in fact find further application in several other systems.

1.6. Mouse adenovirus strain FL.

Much of what has been discussed in the preceding sections concerning the structure of the viral particle and its genome may be applied to the mouse adenovirus. However, since the murine adenovirus was used frequently in the experimental work, a short introduction to the special features of the mouse virus is appropriate at this stage.
Mouse adenovirus strain FL (AdFL) was originally isolated by Hartley and Rowe (1960) and classified as an adenovirus on the basis of particle size, resistance to lipid solvents, cytopathic effect and immunological cross-reaction with sera from guinea pigs infected with human adenoviruses. Further characterization of the virus was carried out by Larsen and Nathans (1977) and Wigand et al. (1977) who studied virion architecture as well as certain structural features of the linear DNA genome. The double-stranded DNA molecule has an estimated molecular weight of 19 - 20×10^6 daltons (Larsen and Nathans, 1977; Temple et al., 1981) and is thus smaller than the human adenovirus genome. The DNA is covalently bound to a protein at the 5'-termini which is a feature of all

known adenoviruses (Larsen and Nathans, 1977; Temple et al., 1981). The size of the terminal protein on the mouse adenovirus genome is at present unknown. However, a comparison of the structural proteins from Ad2 and AdFL purified virions by SDS-polyacrylamide gel electrophoresis indicates that the AdFL coat proteins are smaller than their counterparts in Ad2. The largest protein in the AdFL particle, subsequently identified as the major capsid protein or hexon, has an estimated molecular weight of 90K as compared to the 110K hexon protein of Ad2 (Antoine et al., 1982). One might thus perhaps expect the AdFL terminal protein and its precursor to be somewhat smaller in size than those found in the human adenoviruses. Since there is no available transcription map for the AdFL genome, it is not known where the mouse adenovirus terminal protein is coded.

Hybridization studies between mouse AdFL DNA and human Ad2 DNA reveal little or no homology between the two viruses which is in contrast to the internally located regions of homology observed between several human adenovirus serotypes (Tolun et al., 1979). However, there is evidence for antigenic cross-reactivity between the mouse and human adenoviruses which suggests one or more common antigenic determinants on the viral capsid. Recently, two well-conserved regions on the AdFL and Ad2 genomes were recognized (Larsen et al., 1979; Antoine et al., 1982). These two regions of homology have been identified by reference to a functional map of Ad2 and include an extensive DNA sequence homology at map coordinates 52.5 - 60.2, which covers almost the complete gene for the Ad2 hexon protein, and a second region of homology at map coordinates 13.8 - 15.5. This latter conserved region is located in a sequence coding for the IVa_2 protein of Ad2 (hexon associated protein) and also overlaps with a coding sequence for a 120K polypeptide which is a candidate product of the N-gene (Galos et al., 1979). AdFL DNA has an inverted terminal repetition and its primary sequence has also been determined (Temple et al., 1981). Mouse adenovirus ITR consists of 93 base pairs and the repeat shares the terminal 17bp with the group C human adenoviruses. The 10bp sequence (ATAATATACC) described in 1.2.1. is almost perfectly conserved in AdFL and the mouse genome also shares a stretch of 6bp

from position 9 to 14 with all known adenoviruses (Aleström et al., 1982).

Mouse adenovirus FL has a limited host range (Larsen and Nathans, 1977; Dussaix et al., 1982). For all practical purposes, human HeLa cells represent a non-permissive host for AdFL virus and the yield of infectious viral particles in HeLa cells is reduced by at least 2000-fold as compared with permissive mouse 3T3 cells (Antoine et al., 1982). However, after infection of HeLa cells with AdFL, the rate of viral DNA synthesis is comparable to that in the permissive AdFL/3T3 system, the only difference being that the maximum of DNA synthesis is reached 11 hours later in the AdFL/HeLa system after an initial delay. The defect in virus production in this heterologous system was linked to a dramatic reduction in the synthesis of AdFL structural proteins and in particular, hexon synthesis. The molecular basis of this block in structural protein synthesis is not known. This AdFL/HeLa cell system may be compared to other adenovirus/host cell heterologous systems e.g. human adenovirus/monkey cell (Rabson et al., 1964; Klessig and Chow, 1980) and human adenovirus/CHO (hamster) cell (Longiaru and Horwitz, 1981). In all these heterologous systems, the extent of viral DNA synthesis is indistinguishable from that in the homologous mouse system. These observations clearly indicate that early AdFL genes are being expressed and thus, the synthesis of the early viral proteins and viral induced protein participating in AdFL DNA replication proceeds apparently unimpaired in the "foreign" host cell.

2. MATERIALS .

2.1. Abbreviations.

Ad	adenovirus
AdFL	mouse adenovirus strain FL
AdX	adenovirus serotype X
bp	base pairs
Ci	curie (1Ci = 3.7×10^{10} Becquerels)
cpm	counts per minute
ddNTP	dideoxyribonucleoside triphosphate
dNTP	deoxyribonucleoside triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ds	double-strand
DTT	dithiothreitol (Cleland's reagent)
EDTA	ethylenediaminetetracetic acid
ffu/ml	focus forming units per millilitre
fig.	figure
g	gravity
g/l	gram per litre
GuHCl	guanidinium hydrochloride
h	hour
hpi	hour post infection
HEPES	N-2-hydroxyethylpiperazine- N'-2-ethanesulfonic acid (pH adjusted with KOH)
K	kilodalton
M	molar
mA	milliampere
min	min
mg	milligram
OD_x	optical density at the wavelength x in nm
PAA	polyacrylamide
PBS-d	phospho-buffered saline without Ca^{2+} or Mg^{2+}
pfu/ml	plaque forming units per millilitre
PMSF	phenylmethylsulfonyl fluoride

RIA-BSA	radio-immuno-assayed bovine serum albumin
RNA	ribonucleic acid
rpm	revolutions per minute
sec	second
SDS	sodium dodecyl sulphate
ss	single-strand
TCA	trichloroacetic acid
TE	Tris buffer/EDTA solution
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris-(hydroxymethyl)-aminomethane
ts	temperature-sensitive
UV-light	ultra-violet light
vol.	volume

2.2. Cells.

HeLa cells were a gift from Dr. J. Williams, Glasgow, Scotland.
A549 cells were obtained from Dr. T. Shenk, Stony Brook, New York, USA.
Mouse 3T3 cells (BALB/c and NIH) were provided by G. Antoine and Dr. M. Lipp, München, FRG.

2.3. Virus.

Adenovirus 2 was obtained originally from Dr. W. Rowe, NIH, Bethesda, Maryland, USA.
Adenovirus FL was a gift from G. Antoine, München, FRG.

2.4. Sera.

Newborn calf serum was obtained from Flow Laboratories, Bonn, FRG.
Foetal calf serum was from Seromed, München, FRG.

2.5. Bacteria.

E.coli (type JM83) were grown in L-broth: 10g bacto-tryptone, 5g yeast extract and 5g NaCl per 1 litre of medium. The medium was prepared with didistilled water and autoclaved for 40-60min at 120°C before use.

2.6. Chemicals and other substances.

Various reagents were purchased as follows:

Amersham und Buchler, Braunschweig, FRG:
^{14}C-sodium formate (50-60mCi/mmole);
deoxycytidine-5'-(-^{32}P)-triphosphate

Baker, J.T. Chemical Company, Philipsburg, New Jersey, USA:
cesium chloride (CsCl), glycerol

BDH Chemicals Ltd., England:
Aristar guanidinium hydrochloride, Analar sucrose

Bethesda Research Laboratories Inc. (BRL), Maryland, USA:
agarose, restriction endonuclease Hind III

Bio-rad Laboratories, Richmond, Calif., USA:
sodium dodecyl sulphate (SDS)

Boehringer, Mannheim, FRG:
adenosine-5-triphosphate (ATP), the four dNTPs (dATP, dTTP, dCTP, dGTP), dideoxyATP (ddATP), dideoxyGTP (ddGTP), chloramphenicol, restriction endonuclease Eco RI

Calbiochem-Behring Corp., La Jolla, Calif., USA:
dithiothreitol (Cleland's reagent)

Difco Laboratories, Detroit, Michigan, USA:
bacto-trytone, yeast extract

Flow Laboraties, Bonn, FRG:
Gentamicin

Kodak Limited:
X-ray films, D19 developer

LKB, France:
Ultrogel A6

Merck, E. Darmstadt:
acetic acid, ammonium peroxodisulfate, bromophenol blue, diethyl ether, dimethylsulfoxide (DMSO), EDTA, ethanol, glucose, glycine, hydrochloric acid, isopropanol, magnesium chloride, methanol, phenol, potassium chloride, proteinase K from fungus, sodium acetate, sodium chloride, TEMED, trichloroacetic acid, triton-X-100

Nunc GmbH., Wiesbaden, FRG:
cell culture vessels

Scharz/Mann, Orangeburg, New York, USA:
ammonium sulphate

Serva, Heidelberg, FRG:
acrylamide, dialysis tubing, glass wool, HEPES, hydroxyurea, ß-mercaptoethanol, N,N'-methylene bisacrylamide, phenylmethyl-sulfonyl fluoride (PMSF), 1,1,2-trichlorotrifluoroethane (Uvasol)

Sigma, München, FRG:
agarose, ampicillin, ethidium bromide,
Tris-(hydroxymethyl)-aminomethane (Tris), RIA-bovine serum albumin

Whatmann Ltd., Kent, USA:
GF/C filters

Zinsser, Frankfurt, FRG:
Quickzint 501

Aphidicolin was a gift from Dr. A.H. Todd, ICI, England.

The restriction endonuclease Xba I was isolated by the author prior to this work, basically as described by Zain and Roberts (1977).

2.7. Buffers and other solutions.

ampicillin : sodium salt of ampicillin, 50mg/ml in water
aphidicolin : stock solution approx. 80mM in DMSO, dilutions in water
BND-A : 250mM NaCl, 10mM Tris-HCl pH 8.0, 5mM EDTA pH 8.0
buffer G (plasmid preparation) : 10mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0, 10mM NaCl
buffer N (purification of nuclear extracts) : 50mM HEPES pH 7.5, 100mM NaCl, 2mM ß-mercaptoethanol, 10% sucrose
Dulbecco's medium : as described by Dulbecco and Freeman (1959)
Eagle's medium (modified) : as described by Winnacker, (1975)
electrophoresis buffer for agarose gels : 40mM Tris-acetate pH 7.8, 5mM Na-acetate, 1mM EDTA pH 8.0
electrophoresis buffer for SDS-PAA gels : 25mM Tris base, 192mM glycine, 0.1% SDS
Hoechst stain 33258 : (2-[2-(4-hydroxyphenyl)-6-benzimidazolyl] -6-(1-methyl-4-piperazyl)-benzimidazol-3HCl) stock solution 0.1mg/ml in methanol
hypotonic HEPES buffer (preparation of nuclei) : 20mM HEPES pH 7.5, 5mM KCL, 0.5mM $MgCl_2$, 0.5mM DTT
isotonic HEPES buffer (preparation of nuclei) : 50mM HEPES pH 7.5, 10% sucrose
lysis mix (bacteria) : 50mM Tris-HCl pH 8.0, 62.5mM EDTA pH 8.0, 0.2% triton-X-100
McIlvane buffer : 0.1M citric acid, 0.2M Na_2HPO_4
PBS-d : phospho-buffered saline without Ca^{2+} or Mg^{2+} as described by Fütterer, 1982
PMSF : stock solution 100mM in ethanol or isopropanol

restriction enzyme buffers : Eco RI: 100mM Tris-HCl, 50mM NaCl, 5mM $MgCl_2$; Hind III: 7mM Tris-HCl pH 7.5, 60mM NaCl, 7mM$MgCl_2$; Xba I: 6mM Tris-HCl pH 7.8, 150mM NaCl, 6mM $MgCl_2$

TE : 10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0

Tris-saline solution : as described by Fütterer, 1982

sample buffer for PAA-SDS samples (3-fold concentrated) : 150mM Tris-HCl pH 6.8, 30% glycerol, 9% ß-mercaptoethanol, 9% SDS, 0.006% bromophenol blue

All solutions were prepared in didistilled water unless otherwise stated.

3. METHODS .

3.1. Cell culture.

3.1.1. HeLa cells.

HeLa cells were grown in monolayer and suspension cultures at 37°C. The monolayer cultures were grown in Dulbecco's medium (Dulbecco and Freeman, 1959) in an atmosphere of 5% CO_2 / 95% air and the suspension cultures were grown in a modified Eagle's medium with constant stirring (Eagle, 1959). Both media were supplemented with 10% newborn calf serum. In addition, gentamycin was included in all media at a final concentration of 50µg/ml to prevent the growth of various species of mycoplasma. The monolayer HeLa cells were passaged every 5 - 7 days basically as described by Kathmann (1974). Medium was removed and the cells were washed twice with PBS-d. About 1ml of a trypsin solution was added to each flask and, after a brief incubation to release the cells in the monolayer, the cells were collected in about 1ml of newborn calf serum by centrifugation (3500rpm, 10min). The cells were passaged at a dilution of 1:6.

3.1.2. A549 cells.

A549 cells, derived from a human lung carcinoma, were maintained in monolayer cultures using Dulbecco's medium supplemented with 10% foetal calf serum.

3.1.3. 3T3 cells.

Mouse 3T3 cells (BALB/c or NIH) were grown as monolayer cultures in plastic Falcon flasks (80cm^2) or in large culture plates (600cm^2). The culture medium was as described for HeLa cells but 3T3 cell passage was more frequent i.e. at about 2/3 confluency every 3 - 4 days.

3.2. Mycoplasma test.

All monolayer cultures were tested regularly for mycoplasma contamination using a modification of the method of Chen (1977). Cells were grown on sterile coverslips (15 x 15mm) in 35mm (diameter) petri-dishes to semi-confluency (usually overnight cultures). The medium was removed and the cultures were washed once with PBS-d. The stock solution of the fluorescent stain, Hoechst 33258 (see Materials), was diluted 1:100 in methanol just before use (stock solution 0.1mg/ml) and 2ml of this dilution were added to each petri-dish. The cultures were left for 30 minutes at room temperature, after which time the stain was removed and the cultures were washed once with PBS-d. The coverslip was then embedded in one drop of 1:1 glycerol:McIlvane buffer (0.1M citric acid, 0.2M Na_2HPO_4 pH 5.5) and the cells were examined under oil in a fluorescent microscope (Leitz). The chemical stains DNA and contaminated cultures showed fluorescent mycoplasma within the cells and near cellular membranes.

3.3 Growth of adenovirus.

3.3.1. Ad2 on HeLa cell monolayers.

This method was used for the production of ^{14}C-labelled adenovirus and for making adenovirus stocks. Almost confluent HeLa cells growing in Falcon flasks ($0.5\text{-}1.0 \times 10^7$ cells/80cm^2 flask) were washed once with 1/2 tsd (a mixture of one part Dulbecco's medium and one part Tris-saline). The adenovirus stock, suitably diluted with 1/2 tsd, was then added to the cultures at a multiplicity of 10-20 pfu/cell. About 3ml virus solution were added to each flask and the cells were incubated for 1-2h at 37°C. Dulbecco's medium, supplemented with 2% newborn calf serum, was then added (40ml per flask) and the incubation was continued at 37°C.

For the production of ^{14}C-labelled virus, the medium was reduced

to about 15ml in all flasks and ^{14}C-formic acid (5 - 15µCi/ml) was added at 6-8h post infection.
After the appearance of a cytopathic effect, 36-46h post infection, the medium was removed and 1ml of 0.02M Tris-HCl pH 8.0 was added to each flask. The cells were then frozen at -20°C and subjected to three cycles of freezing and thawing. The resulting lysate consisted of a crude preparation of virus and was stored at -20°C for use in further infections.

To purify the ^{14}C-labelled virus, the lysate was sonicated using a Branson sonifier (4 x 30sec/65mA) and, after removal of cell debris by centrifugation (bench centrifuge, 3500rpm, 10min, 4°C), the supernatant was extracted twice with 1,1,2-trichlorotrifluoroethane (Uvasol). The crude virus preparation was then purified by centrifuging to equilibrium in CsCl gradients as follows: X/2g CsCl was added to Xml virus suspension and the gradients were centrifuged for at least 2h at 47000rpm at 4°C in a vertical rotor (Sorvall ultracentrifuge, rotor TV 865). The virus particles appear as a white band in the gradient at a density of 1.334g/cm^3 and this band was easily removed using a pasteur pipette or by collecting gradient fractions. The centrifugation step was repeated twice and the virus was stored in CsCl for several weeks at 4°C.
To determine the amount of ^{14}C-label incorporated into the virus, an aliquot (10µl) of the CsCl-virus suspension was precipitated with 5% TCA on ice for 30min. Unlabelled bovine serum albumin was added as carrier (about 250µg protein). TCA-insoluble material was collected on GF/C-filters (Whatman) and after addition of a toluol-based scintillation fluid (Quickzint 501, Zinsser), the samples were counted in a liquid scintillation counter.

3.3.2. Ad2 in HeLa cell suspension cultures.

HeLa cells in suspension culture were infected with adenovirus stocks (10-20 pfu/cell) at a cell density of 2x10^{6}cells/ml. Virus was allowed to adsorb for 1-2h and the culture was then diluted 10-fold with Eagle's medium supplemented with 2% newborn calf serum

and stirred for 40-48h at 37°C. The cells were pelleted by centrifugation (1200rpm, 4°C, 10min) and the pellet was resuspended in 10-20ml 0.02M Tris-HCl pH 8.0. CsCl-purified virus was prepared from this suspension as described in 3.3.1. for ^{14}C-labelled virus.

3.3.3. AdFL on 3T3 cell monolayers.

Semi-confluent mouse 3T3 cell monolayers ($80cm^2$ or $600cm^2$ culture vessels) were washed once with 1/2 tsd. AdFL virus stock was suitably diluted with 1/2 tsd and added at a multiplicity of 0.5 - 1.5ffu/cell to the monolayers. After a 1h adsorption period, Dulbecco's medium supplemented with 2% newborn calf serum was added and the cultures were incubated at 37°C for 3 - 4 days. A cytopathic effect was then visible. The medium was removed and 2ml Dulbecco's were added (10ml to the large $600cm^2$ plates). The cells were subjected to a freeze/thaw procedure and the resulting lysate was used directly as stock virus for further infections.

3.4. Determination of virus titres.

Two different methods were used to determine the virus titres for the human and mouse adenovirus species. Adenovirus type 2 titres were determined by plaque tests using A549 cells and adenovirus FL titres were determined by immunofluorescence in 3T3 cells.

3.4.1. Plaque tests with Ad2.

This method was modified from Williams (1970). Just confluent A549 cell monolayers in petri-dishes (35mm diameter) were infected with a series of stock virus dilutions (10^0 - 10^{-10}) in 1/2 tsd. After adsorbtion for 1h, the virus solution was removed and an overlay consisting of Dulbecco's medium, 0.8% agarose, 2.5% foetal calf serum and 50mM $MgCl_2$, cooled to 40 - 45°C, was applied. Incubation was carried out at 37°C for 4 - 5 days. A second overlay of the same composition but supplemented with 0.02% neutral red was

then added. After a further 12 - 20h incubation at 37°C, plaques were counted.

3.4.2. Immunofluorescence with AdFL.

Immunofluorescent detection of viral antigens was performed essentially as described by Vogt (1969). 3T3 cell monolayers were grown on sterile coverslips (15mm x 15mm) in petri-dishes (35mm diameter) to semi-confluency, usually overnight cultures. Medium was removed and the cells were infected with a series of stock virus dilutions (10^0 - 10^{-7}) in 1/2 tsd. After adsorption for 1h at 37°C, the virus solution was removed and Dulbecco's medium supplemented with 2% newborn calf serum was added. Incubation was continued at 37°C for 3-4 days. On day three, medium was removed, the cells were washed once with cold Tris-saline and fixed with 2ml ice-cold absolute ethanol at -80°C for 30min. The cultures were then washed again with Tris-saline and the cover-slips were transferred to fresh petri-dishes. A stock anti-AdFL solution (antibody to mouse virus coat proteins prepared from infected rabbit serum) was diluted 1:30 with Tris-saline and approximately 50µl were added to each cover-slip culture. The cells were then incubated for 1h at 37°C. Unbound anti-AdFL antibody was removed by washing extensively with Tris-saline (3-4 times) followed by the addition of 50µl of anti-rabbit serum (stock solution diluted 1:20 with Tris-saline) containing covalently-bound FLUORESCEIN to all cultures and these were incubated again for 1h at 37°C. Finally, the cells were washed with Tris-saline (3 times) and the coverslips were embedded in one drop of glycerol/McIlvane buffer (see 3.2.). The cultures were then examined under a fluorescent microscope and fluorescent cells were counted in at least five different areas on the cover-slip. The virus titre was determined, taking account of the petri-dish area and the virus dilution used for infection, and the result was expressed as focus forming units per millilitre crude virus suspension (ffu/ml). In general, the virus titre determination was repeated on day four after infection using duplicate cover-slip cultures. Since the AdFL virus reproduction cycle is relatively long in mouse 3T3 cells, a mean value for the

virus titre was obtained from immunofluorescent tests on day three and day four after infection.

3.5. Isolation of adenovirus DNA-protein complex.

Adenovirus DNA-protein complex was isolated by a modification of the method of Sharp et al. (1976). Virus was purified by three successive bandings in CsCl (3.3.1.) and dialysed against 400ml of 10mM Tris-HCl, 1mM EDTA pH 8.0 (TE) containing 1mM PMSF for 1h at 4°C. After dialysis, virions were disrupted with an equal volume of 8M guanidinium hydrochloride (GuHCl in TE/1mM PMSF) and incubated on ice for 10 - 20 minutes. The mixture was then applied to a linear sucrose gradient (5 - 20% sucrose in 4M GuHCl/TE/1mM PMSF) and DNA-protein complex was separated from non-covalently bound protein by centrifugation at 30000rpm in a SW41-rotor for 16h at 4°C (Sorvall ultracentrifuge). The sucrose gradients were poured in nitrocellulose centrifuge tubes which were previously incubated with RIA-BSA (0.5mg/ml in TE/1mM PMSF) for 30min at 55°C, rinsed several times with distilled water and dried at 80°C. After centrifugation the gradients were collected in approximately 0.5ml fractions at 4°C and RIA-BSA was added to each fraction to a final concentration of 0.5mg/ml. Agarose gel electrophoresis of fraction aliquots and subsequent staining with ethidium bromide (3.9.2.) allowed determination of DNA-containing gradient fractions. Fractions with the maximal DNA content were pooled and dialysed against several changes of TE/1mM PMSF for 48 - 72h at 4°C. After dialysis, the DNA content of the samples was determined by measurement of the optical density at 260/280nm and had a mean value of 40 - 50µg/ml.

CsCl-purified mouse AdFL virus was a gift from G. Antoine. Viral DNA-protein complex from AdFL virus was prepared as described above. Both adenovirus DNA-protein complexes were stable at 4°C for several months.

3.6. Preparation of adenovirus DNA.

Adenovirus in CsCl (3.3.1.) was dialysed for 2h against BND-A buffer at 4°C and then incubated with proteinase K (200µg/ml) in the presence of 0.5% SDS for 2h at 37°C. Protein was separated from DNA by extracting 3 times with phenol saturated with TE (pH 7.5). The aqueous phase containing the DNA was dialysed against BND-A buffer (2h) and then against several changes of 10mM Tris-HCl pH 7.5 and 50mM NaCl (48h). The concentration of DNA was determined by measuring the absorbance at 260nm and at 280nm (OD_{260} = 20 represents 1mg DNA/ml).
Protease-treated AdFL DNA was a gift from G. Antoine.

3.6.1. Digestion of DNA with restriction enzymes.

Adenovirus DNA or DNA-protein complex was digested with the restriction enzyme Xba I (Xanthomonas badrii). Digests were carried out in sterile, siliconised Eppendorf tubes and contained in a total volume of 70 - 100µl approximately 2 - 4µg DNA (or DNA-protein complex), 5 - 10µl of the enzyme Xba I (partially purified preparation, see Materials) and reaction buffer which was 6mM Tris-HCl pH 7.8, 6mM $MgCl_2$ and 150mM NaCl. The samples were incubated at 37°C until fully digested (about 2 - 6 hours) as judged by agarose gel electrophoresis (3.9.2.). The digested DNAs were then stored without further additions at 4°C until required.
Supercoiled plasmid DNA, prepared as described in 3.12.1. was linearized with the restriction enzyme Eco RI (Escherichia coli). About 10 - 50µg DNA were digested in a total volume of 100 - 500µl containing Eco RI (about 2 Units per µg DNA) and reaction buffer which was 100mM Tris-HCl pH 7.5, 5mM $MgCl_2$ and 50mM NaCl. After complete digestion at 37°C, protein was separated from the DNA by extracting at least three times with TE-saturated phenol. The aqueous phase containing the DNA was then freed of phenol by extracting three times with ether which was, in turn, removed by a short dialysis or by allowing the ether to evaporate in a warm water-bath. Finally, the aqueous phase was transferred to a new Eppendorf tube and DNA was precipitated with 3M Na-acetate (about

1/10 volume) and ice-cold 100% ethanol (about 2 - 3 volumes) at -80°C for 1 - 2 hours. The pellet was washed several times with ice-cold 80% ethanol and air-dried. The DNA was then resuspended in TE (10:1) at a final concentration of 0.5 - 1mg/ml and stored at 4°C.

3.7. Preparation of nuclei from adenovirus infected cells.

3.7.1. Ad2 infected HeLa cells.

HeLa cells, maintained in suspension culture (1 - 2 litres), were infected with crude preparations of Ad2 (approx. 20 - 100pfu/cell) at a density of 3 - $5x10^5$ cells/ml as described in 3.3.2. At 2h after infection, hydroxyurea was added to a final concentration of 10mM. The cells were harvested at 20 - 22h post infection by centrifugation at 1200rpm for 10min at 4°C and washed three times in ice-cold hypotonic buffer containing 0.2M sucrose. The cells were then resuspended in cold hypotonic buffer (about 15ml) and allowed to swell for 10 - 20min on ice. Finally, the cells were lysed by 10 strokes of a tight-fitting Dounce homogenizer (Braun Melsungen). An aliquot of the suspension was examined by phase contrast microscopy to determine the extent of cell-lysis and, if necessary, the lysis procedure was repeated. The resulting lysate was centrifuged in a 15ml glass tube at 3500rpm (Labofuge II, Heraeus Christ) for 15min at 4°C. The supernatant containing the cytoplasmic fraction was frozen in 2ml aliquots in liquid nitrogen. The nuclear pellet was resuspended in an equal volume (i.e. 1:1 ratio) of isotonic buffer and frozen immediately in 0.2ml aliquots in liquid nitrogen. Both cytoplasmic fractions and nuclei were stored at -80°C.

Uninfected cell nuclei were prepared as described above. The cells were also treated with 10mM hydroxyurea but were not infected with virus.

3.7.2. AdFL infected 3T3 cells.

Mouse 3T3 monolayer cultures were maintained at 37°C in large culture plates (600cm^2). Infection of semi-confluent 3T3 cells was carried out as described in 3.3.3. except that cells were infected with a multiplicity of 3 - 6ffu/cell. At 6 - 7hpi, hydroxyurea was added to a final concentration of 2.5 - 5mM. The cells were harvested at 40 - 45hpi by scraping the plates with a sterile rubber policeman. The plates were washed once with ice-cold hypotonic buffer containing 0.2M sucrose and the combined cell suspensions were then washed three times in the same buffer. The nuclei were prepared as described above for Ad2 infected HeLa cells and cytoplasmic fractions and nuclei were frozen in liquid nitrogen and stored at -80°C.

3.7.3. AdFL infected HeLa cells.

HeLa cells in suspension culture (1 litre) were infected with crude preparations of AdFL at a multiplicity of 3 - 6ffu/cell. Hydroxyurea was added to a final concentration of 10mM at 7h post infection. Cells were harvested at 50 - 55hpi and nuclei were prepared as described above for Ad2 infected HeLa cells. As before, the nuclei and cytoplasmic fractions were stored at -80°C.

3.8. Preparation of extracts.

3.8.1. Nuclear extracts.

HeLa cell nuclei or 3T3 cell nuclei infected with adenovirus (Ad2 or AdFL) were allowed to thaw slowly on ice (about 1h). Extracts were prepared by adding NaCl to a final concentration of 100 - 150mM (HeLa) or 200mM (3T3) and the nuclei were then incubated for 1h on ice. After centrifugation at 12000 rpm in an Eppendorf centrifuge for 15min at 4°C, the clear non-viscous supernatant was removed with a pasteur pipette into a sterile Eppendorf tube and kept on ice until needed for *in vitro* experiments.

Occasionally, nuclear extracts were partially purified by ammonium sulphate precipitation. The infected cell nuclei were extracted with salt as described above and proteins were then precipitated in a 15ml corex tube by 40% ammonium sulphate which was added slowly at 4°C (0.226g added to 1ml of solution). The precipitate was collected by centrifugation at 5000g for 20min at 4°C (Sorvall centrifuge, rotor SS34). After removal of the supernatant, the precipitate was resuspended in a minimal volume (circa 150µl per 1ml original solution) of buffer N (50mM HEPES/KOH, pH 7.5; 100mM NaCl; 2mM ß-mercaptoethanol; 10% sucrose) and dialysed overnight against 2 x 0.25l of buffer N at 4°C. On the following day, a whitish precipitate which formed during dialysis was removed by centrifugation at 12000rpm for 3min at 4°C. The remaining supernatant containing concentrated nuclear proteins and other factors was frozen immediately in liquid nitrogen and stored at -80°C.

3.8.2. Cytoplasmic extracts.

Infected or uninfected cytoplasmic fractions (HeLa or 3T3) were thawed and then centrifuged for 15min at 12000 rpm (4°C) to remove any contaminating nuclei. The supernatant was then removed and kept on ice until required.

3.9. Gel electrophoresis.

3.9.1. PAA gels.

Proteins were separated on SDS-polyacrylamide gels according to the method of Laemmli (1970). The gels were, in general, 12cm long and 1.5mm thick. The separating gel contained 0.377M Tris-HCl pH 8.8; 0.1% SDS and 10% acrylamide. The ratio of acrylamide to bis-acrylamide was 30:0.4. The mixture was degassed and then polymerised with 25µl TEMED (N,N,N',N'-tetramethylethylenediamine) and 250µl ammonium persulphate (10% in water). After pouring the gel, a few millilitres of water:isopropanol (1:1) were layered on

top to obtain a smooth surface. The stacking gel (about 2cm) consisted of 0.125M Tris-HCl pH 6.8; 0.1% SDS and 5% acrylamide (30:0.4). The mixture was degassed and polymerised with ammonium persulphate (150µl) and TEMED (15µl).

Running buffer for SDS-polyacrylamide gel electrophoresis contained 0.025M Tris base, 0.192M glycine and 0.1% SDS. Gels were run at 15mA/gel until the dye (bromophenol blue) reached the bottom.

3.9.2. Agarose gels.

Agarose gel electrophoresis (Sharp et al., 1973) was used for the analysis of restriction enzyme digested DNA (3.6.1.) and for the location of DNA-protein complex in sucrose gradient fractions (3.5.). The gels were vertical (3mm thick) or horizontal (1 - 3mm thick) and consisted of agarose dissolved in slab gel buffer (40mM Tris-acetate pH 7.8; 5mM Na-acetate; 1mM Na-EDTA pH 8.0). The concentration of agarose varied (0.8 - 1.2%) depending on the size of the fragments to be separated. Sucrose (10%) and bromophenol blue (0.001%) were added to samples before loading. Electrophoresis was carried out at 50V (about 30mA) in slab gel buffer until the bromophenol blue reached the end of the gel. The DNA was visualized by staining the gel with ethidium bromide (2 - 5µg/ml) for 30min and then examining the gel under UV-light (254nm). Gels were photographed with a polaroid camera (film type 107).

3.10. Autoradiography.

Autoradiography was performed on vacuum-dried polyacrylamide gels (3.9.1.) using Kodak X-Omat S X-ray films in Kodak or Dupont cassettes with intensifying screens (Quanta II). Film exposure was at room temperature or at -80°C for various periods of time. The films were developed in Kodak D-19 X-ray developer (5 min, 20 - 22°C), washed briefly and fixed in Superfix (Tetenal) for 5 minutes.

3.11. *In vitro* formation of the initiation complex.

The *in vitro* assay for the formation of the pTP-dCMP complex was a modification of the method originally described by Lichy et al. (1981). The reaction was carried out in a sterile, siliconised Eppendorf tube and contained in a total volume of 25µl:

25mM HEPES pH 7.5 (adjusted with 1M KOH)
2mM DTT
5mM $MgCl_2$
3mM ATP
50µM ddATP
0.6µM dCTP
0.03µM α-^{32}P-dCTP (specific activity 3000 Ci/mmole)
100 - 250µM Aphidicolin (dissolved in DMSO)
100 - 200ng DNA-protein complex
8-13µl nuclear extract

For the formation of the elongated product, ddATP was omitted, dATP and dTTP were added (10-40µM) and the elongation reaction was terminated at the first dG residue by addition of ddGTP at a final concentration of 40µM.
After incubation for 60min at 30°C, the reaction was stopped by addition of sample buffer (50mM Tris-HCl pH 6.8, 10% glycerol, 3% ß-mercaptoethanol, 3% SDS, 0.002% bromophenol blue). Samples were heated for 3min at 100°C and separated by electrophoresis in 10% polyacrylamide-SDS gels. Electrophoresis was carried out at 15mA/gel until the marker dye reached the bottom of the gel (about 9-10 hours later). ^{14}C-labelled adenovirus-2 (3.3.1.) was used as a molecular weight marker and was also heated in sample buffer for 3 min at 100°C (approximately 75000cpm of acid-insoluble material per marker sample). The gels were fixed in several changes of water:methanol:acetic acid (50:40:10) and dried for 2h on a Hoefer Scientific Slab Gel Drier. Autoradiography was performed at room temperature or -80°C with Kodak X-ray films (3.10.).

3.12. Growth of bacteria.

Bacteria containing the required plasmids were grown in L-broth at 37°C with shaking (150 - 180 rpm). Since all plasmids used carried an intact ß-lactamase gene, the medium was supplemented with ampicillin at a final concentration of 50µg/ml. Initial cultures (5ml) were inoculated with the bacterial stock and cells were grown overnight. The cultures were then diluted 1:100 with L-broth and grown to an OD_{600} between 0.7 - 0.8. At this stage, chloramphenicol was added (180mg/l) to stimulate plasmid growth (Clewell, 1972), and incubation was continued overnight.

3.12.1. Isolation of plasmid DNA.

Plasmid DNA was isolated from bacterial cells basically as described by Humphreys et al. (1975). After about 20h growth at 37°C, bacteria were harvested by centrifugation at 6000 rpm (Sorvall centrifuge, GSA rotor) for 10min at 4°C. The cells were suspended in a minimum volume (about 2ml per 250ml culture) of 50mM Tris-HCl pH 8.0 containing 25% sucrose. Lysozyme (stock solution 5mg/ml in 0.25M Tris-HCl pH 8.0) was added (about 0.3ml per 250ml culture) and 0.65ml of 0.2M EDTA pH 8.0. The mixture was incubated on ice for about 5min to disrupt the bacterial cell wall. Then, 2.5ml of a lysis mix (50mM Tris-HCl pH 8.0; 62.5mM EDTA pH 8.0; 0.2% Triton X-100) were added per 250ml culture and the mixture was further incubated on ice to extract plasmid DNA without release of bacterial DNA. As soon as the mixture became slightly viscous (5 - 8min) the cells were separated by centrifugation at 20000 rpm (Sorvall centrifuge, rotor SS34) for 30min at 4°C. Plasmid DNA was purified from the supernatant as follows: 3.325g CsCl were added per 3.5ml lysate in 5ml centrifugation tubes. Ethidium bromide (stock solution 20mg/ml) was added (175µl per tube) and the gradients were centrifuged at 47000 rpm for at least 15h at 17°C (Sorvall ultracentrifuge, rotor TV 865).
Plasmid DNA was visible as a dense band when the gradient was examined with long-wave UV-light (366nm). This band was carefully

removed with a syringe and ethidium bromide was removed by extraction (3 times) with isopropanol (saturated with water and CsCl). The CsCl was then removed by dialysing the DNA against two changes of 10mM Tris-HCl pH 7.5/50mM NaCl overnight at 4°C. The plasmid DNA was further purified by extracting three times with phenol saturated with TE pH 7.5 and finally dialysed extensively against 10mM Tris-HCl pH 7.5; 10mM NaCl; 1mM EDTA at 4°C. The concentration of DNA was determined by measuring the absorbance at 260nm and 280nm (see 3.6.).

Plasmid DNA preparations were freed of contaminating RNA by gel filtration. The gel matrix (Ultrogel A6, LKB; 25,000 - 2,400,000) was suspended in buffer G (10mM Tris-HCl pH 7.5; 1mM EDTA pH 8.0; 10mM NaCl) and a column (12ml volume) was filled with the gel and washed several times with buffer G. Plasmid DNA, supplemented with sucrose and a few drops of bromophenol blue, was added, eluted with buffer G and aliquots of each column fraction were mixed with ethidium bromide (about one drop of a stock solution, 5µg/ml) to locate the DNA. The peak fractions containing DNA were combined, concentrated with alcohol (1/10 vol. 3M Na-acetate; 2 - 3vols. absolute ethanol) for 1 - 2h at -80°C and washed several times with ice-cold 80% ethanol to remove excess salt. The DNA pellet was dried in air and resuspended in a suitable volume of TE.

In general, plasmid preparations contained 0.5 - 2.5mg DNA /l culture depending on the plasmid.

4. RESULTS .

4.1. Introduction.

In order to understand the complex processes involved in the replication of eucaryotic chromosomes, molecular biologists have turned to simple animal viruses whose genomes serve as model systems for studying the replication of DNA. A detailed analysis of cell-free replication systems would provide important data on the molecular mechanisms involved in initiation and chain elongation during DNA synthesis. Further, such systems would provide the means to purify and characterize the proteins involved in DNA replication. At present, the only higher eucaryotic system that initiates DNA synthesis *in vitro* with exogenously-added DNA as template is the adenovirus DNA replication system described originally by Challberg and Kelly (1979a).

The first *in vitro* studies of adenovirus DNA replication were performed with isolated nuclei or subnuclear replication complexes from adenovirus infected cells (Sussenbach and van der Vliet, 1972; Winnacker, 1975; Yamashita et al., 1975; Brison et al., 1977; Kaplan et al., 1977). However, the "replication" observed in these systems represents the elongation of nascent strands which have already been initiated *in vivo* prior to the isolation of the nuclei, and thus, de novo synthesis of DNA does not occur. Over the last few years, soluble enzyme systems have been developed that are capable of replicating exogenously-added adenovirus DNA (Challberg and Kelly, 1979a, b; Kaplan et al., 1979; Ikeda et al., 1980; Reiter et al., 1980;). These *in vitro* replication systems closely resemble the replication of adenovirus *in vivo* . Replication is initiated at either terminus of the genome and proceeds via type I intermediates to produce full length virus DNA. Thus, adenovirus replication *in vitro* , as *in vivo* , occurs semi-conservatively and by a displacement mechanism.

4.1.1. Replication of adenovirus *in vitro* .

The *in vitro* replication system described by Challberg and Kelly (1979a) requires a low-salt nuclear extract from adenovirus infected cells, adenovirus DNA-protein complex as exogenously-added template, the four deoxyribonucleotide triphosphates, a reducing agent to protect sulfhydryl groups, ATP and Mg^{2+}. The reaction product consists of newly synthesized DNA strands predominantly of genomic length that are hydrogen-bonded, but not covalently linked, to the input DNA template. After a 2 hour *in vitro* reaction at 37°C, the newly synthesized DNA cosediments with the input DNA template in neutral and alkaline sucrose gradients. It has been demonstrated that the 5'-termini of nascent adenovirus DNA strands synthesized in this system are covalently linked to the 80K precursor terminal protein (pTP) by a phosphodiester bond (Challberg et al., 1980; Stillman, 1981). Recently, Lichy et al., (1981) have developed a modification of this *in vitro* system to study the initiation of adenovirus DNA replication in the absence of chain elongation. Under these conditions, a covalent complex is formed between the 80000 dalton protein (pTP) and dCMP. This complex may be experimentally detected by incubating adenovirus infected cell extracts with (α-^{32}P)-dCTP as the only dNTP, and examining the labelled product by SDS-polyacrylamide gel electrophoresis. The formation of this protein-nucleotide complex *in vitro* thus supports the protein-priming model for initiation of adenovirus DNA replication (see 1.4.).

During the last few years, several authors have used different variations of the *in vitro* initiation reaction (Lichy et al., 1981; Pincus et al., 1981; Tamanoi and Stillman, 1982; van Bergen et al., 1983). Therefore, it was decided to perform some preliminary experiments in order to determine the optimal conditions for the *in vitro* complex formation assay.

4.2. Preparation of nuclear extracts from adenovirus infected cells.

Adenovirus DNA replication occurs in the nuclei of infected cells. Synthesis of Ad2 DNA begins at 8 - 10 hpi in permissive HeLa cells and signals the switch from the "early" to the "late" phase in viral gene expression. In this system, viral DNA synthesis reaches a maximum at 18 - 22 hpi (Philipson and Lindberg, 1974; Winnacker, 1978; Flint, 1981). Isolated nuclei from infected cells are thus a convenient source of the proteins required for viral DNA replication.

Nuclei were prepared from Ad2 infected HeLa cells at 20 - 22 hpi which, based on the viral DNA synthesis maximum, was the most suitable time to obtain active nuclear extracts rich in replication proteins (Challberg and Kelly, 1979a).

The maximum of mouse AdFL DNA synthesis in mouse 3T3 cells occurs at approximately 40 hpi. Consequently, nuclei were prepared from AdFL infected 3T3 cells as described in 3.7.2. at 40 - 45 hpi to obtain optimal activity in nuclear extracts.

In a less permissive adenovirus/host cell system, it is essential to determine the time course of viral DNA synthesis before isolation of infected cell nuclei. Antoine et al. (1982) have shown that, after an initial eclipse period, AdFL DNA is synthesized efficiently in human HeLa cells and reaches a maximum at approximately 50 hpi. Accordingly, nuclei were prepared at 50 - 55 hpi from this heterologous AdFL/HeLa cell system.

In all cases, nuclei were prepared basically as described by Challberg and Kelly (1979a). The isolated nuclei were frozen immediately in liquid nitrogen and could be stored at -80°C for several months. Replication proteins and other factors were obtained from the nuclei by extraction with 100 - 150mM salt (NaCl) for 1h on ice. In general, 3T3 cell nuclei were extracted with higher salt concentrations (200mM NaCl) as experience showed that higher salt levels gave better results with regard to activity in the mouse *in vitro* initiation assay.

4.2.1. Influence of hydroxyurea on the *in vitro* activity of nuclear extracts.

Since the onset of adenovirus DNA replication in infected cells occurs before the expression of late viral genes, one can assume that sufficient viral proteins and viral induced proteins are produced during the early phase of infection to allow DNA replication to take place. Conditions that inhibit viral DNA synthesis *in vivo* and thus prevent the expression of late viral genes include treatment of infected cells with hydroxyurea or cytosine arabinoside (Ara C) or infection with DNA-negative, temperature-sensitive mutants at the non-permissive temperature (Green et al., 1978). Hydroxyurea has been shown to block the synthesis of cellular and viral DNAs while permitting the synthesis of RNA and protein (Sussenbach and van der Vliet,1973; Yamaguchi et al., 1977). The drug inhibits the enzyme ribonucleotide reductase and thus quickly exhausts the endogenous pool of deoxyribonucleotides in the cell (Young and Hodas, 1964). Hydroxyurea should therefore inhibit the *in vivo* replication of the virus and, at the same time, allow the accumulation of cell and early viral proteins.

HeLa cells were grown and infected with adenovirus as described in 3.7.1. At 2 hpi hydroxyurea was added to final concentrations between 0 - 10mM. The respective nuclear extracts were then tested for complex formation activity *in vitro* as described in 3.11. As shown in figure 4, 10mM hydroxyurea appears to be the optimal drug concentration for complex formation activity in the initiation assay. In the absence of the drug, the extracts are only weakly active. The labelled material at the top of the gel (lane 2) probably represents a high background of *in vitro* synthesis on partially replicated endogenous viral DNA templates. Because of the toxicity of hydroxyurea, the concentration of the drug in infected cells was not increased above 10mM.

In the case of AdFL infected HeLa or mouse 3T3 cells, hydroxyurea was added at 6 - 7 hpi due to the longer eclipse period of viral replication in these cells. Hydroxyurea is toxic for all cells and, in particular, the 3T3 cells seemed to be very sensitive to the

drug. The concentration of hydroxyurea was therefore reduced to 2.5 - 5mM in AdFL infected mouse cells.

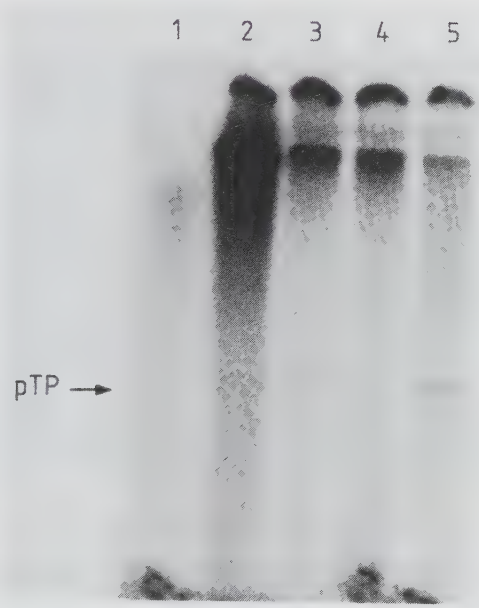


Figure 4. Influence of hydroxyurea on the *in vitro* activity of nuclear extracts.

Nuclear extracts were prepared from Ad2 infected HeLa cells which were treated with various concentrations of hydroxyurea at 2hpi. The extracts were then tested for *in vitro* activity in the standard complex formation assay. Lane 1 is a control without DNA and lanes 2-5 contain Ad2 DNA-protein complex as template. Concentration of hydroxyurea:- lane 1: 10mM; lane 2: none; lane 3: 2mM; lane 4: 5mM; lane 5: 10mM. The position of the Ad2 initiation complex is indicated by pTP.

4.2.2. Variation of dCTP concentration in the *in vitro* assay.

The optimal dCTP concentration in the reaction mixture was determined by preparing a series of reaction mixes containing dCTP at a final assay concentration which varied from 0.3µM to 5µM. The results in the initiation assay, figure 5, lanes 2-6, show that increasing the molar dCTP concentration above 1.2µM significantly reduces the incorporation of α-^{32}P-dCTP and thus the labelled protein band at 87K becomes less apparent. Reaction mixtures therefore contained dCTP at an assay concentration of 0.6µM in all further tests.
The specific activity of α-^{32}P-dCTP was approximately 3000 Ci/mmole. About 2µCi were included in each assay which resulted in a final concentration of approximately 0.03µM α-^{32}P-dCTP in addition to the unlabelled dCTP present.

4.2.3. Omission of ATP in the initiation reaction.

The dependence of complex formation on ATP was investigated. In the absence of exogenously-added ATP, the amount of pTP-dCMP complex formed was drastically reduced, figure 5, lane 8. The omission of ATP from the reaction mixture also gave rise to one or more labelled bands not observed in the presence of ATP. The nature of these bands was not investigated but they probably represent the unspecific incorporation of label caused by the lack of ATP in the reaction mixture. Possible functions of ATP in complex formation are discussed in 5.2.; however, the precise role of ATP in the initiation of adenovirus DNA replication remains unclear.

4.2.4. Omission of ddATP in the initiation reaction.

The pTP-dCMP complex formation reaction was carried out as described in 3.11. but dideoxyATP (ddATP), which is normally present at a concentration of 50µM, was omitted. The absence of ddATP had no significant effect on the amount of complex formed but the label at the top of the gel was slightly increased (figure 5, lane 9). Since it lacks a 3'-hydroxyl group on the sugar residue,

ddATP prevents further elongation when incorporated into a DNA chain. Thus, extensive incorporation of label by a repair-like reaction should be prevented by the presence of excess ddATP in the in vitro reaction.

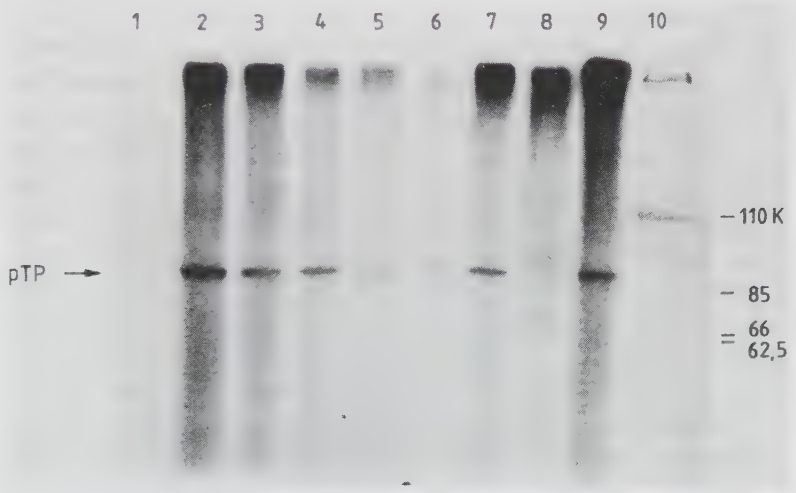


Figure 5. Optimizing conditions for the formation of the initiation complex in vitro .

Lanes 2-9 all contain Ad2 DNA-protein complex as template and lane 1 is a control without DNA. The in vitro reactions were performed as described in 3.11. with the following modifications:- lane 1: no DNA; lane 2: 0.3µM dCTP; lane 3: 0.6µM dCTP; lane 4: 1.2µM dCTP; lane 5: 2.5µM dCTP; lane 6: 5µM dCTP; lane 7: nuclear extract incubated at 30°C for 30min before use; lane 8: no ATP; lane 9: no ddATP. Lane 10 contains ^{14}C-labelled adenovirus 2 marker proteins with the respective molecular weights shown at the right in kilodaltons (K). The arrow indicates the position of the pTP-dCMP complex.

4.2.5. Preincubation of nuclear extracts.

Nuclear extracts from Ad2 infected HeLa cells were incubated at 30°C for 30min immediately before use. This treatment should help to reduce a background resulting from a repair-like reaction

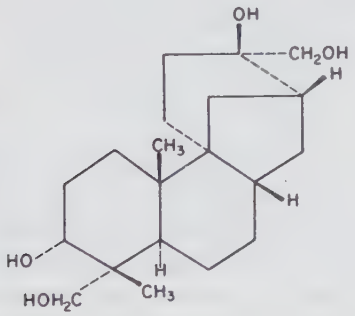
(Challberg et al., 1982). However, no significant difference was observed for most extracts in the *in vitro* initiation assay (figure 5, lane 7) and in general, nuclear extracts were used directly in the assay without preincubation.

4.2.6. Aphidicolin in the *in vitro* assay.

Aphidicolin is a tetracyclic diterpene tetraol with a steroid-like structure. It is obtained from *Cephalosporium aphidicola* and certain other fungi (Bucknall et al., 1973). The drug inhibits cell division, cellular DNA synthesis and repair replication in many eucaryotic cells (though not in normal yeast cells which are impermeable to it) without affecting RNA or protein synthesis (Huberman, 1981). Aphidicolin is a specific direct inhibitor of animal DNA polymerase α (more than 90% inhibition by 10^{-4}M drug concentration) but it has no detectable effects on polymerases ß and γ or on procaryotic polymerases e.g. DNA polymerases of E.coli or T4 phage (Ikegami et al., 1978; Pedrali-Noy and Spadari, 1980). Adenovirus DNA replication is also sensitive to inhibition by this antibiotic although substantially higher concentrations (300 - 400 fold) are required for complete *in vivo* inhibition than is normally required for complete inhibition of cellular DNA synthesis. Replication of adenovirus *in vitro*, using either subnuclear replication complexes or nuclear extracts from infected cells, is also sensitive to aphidicolin and the drug appears to exert its effect by slowing the overall rate of DNA chain elongation (Longiaru et al., 1979; Kwant and van der Vliet, 1980; Pincus et al., 1981). However, several authors have shown that the initiation of adenovirus DNA replication is unaffected by aphidicolin even at high concentrations e.g. 150µM (Lichy et al., 1981; Pincus et al., 1981). If the initiation reaction is carried out *in vitro* in the presence of aphidicolin (100 - 250µM), the amount of complex formed is not significantly reduced compared to the reaction without aphidicolin. In the absence of the drug however, a high background is obtained and a lot of labelled material accumulates at the top of the gel (not shown). This labelled material presumably represents synthesis of DNA by

polymerase∝ in a repair-like reaction, a process which would be prevented by aphidicolin. Since these experiments were concerned only with the initiation and limited elongation processes of adenovirus DNA synthesis, aphidicolin was included in all assays at a concentration of 100 - 250μM to reduce the background on the polyacrylamide gels.

Aphidicolin was dissolved to approximately 27mg/ml in dimethyl sulfoxide (DMSO) and diluted into water before use. The final concentration of DMSO in the in vitro reaction was less than 0.5% and was not inhibitory.



APHIDICOLIN

4.2.7. The in vitro initiation assay.

The preliminary experiments described in the preceding sections allowed the setting up of a reproducible in vitro assay system for monitoring the initiation of adenovirus DNA replication (3.11.). Many of the assay components were prepared as a concentrated 10-fold mix which was stable for several weeks at -20°C. Extracts from infected cell nuclei were, in general, prepared fresh for each experiment since repeated freezing and thawing of nuclear extracts rapidly reduced in vitro activity.

The reaction was carried out in a minimum volume (25µl) in a sterile, siliconised Eppendorf tube to prevent reactants "sticking" to the walls of the tube. This assay was also used, with slight modifications, for the initiation of mouse adenovirus FL replication *in vitro* (4.3.4.)

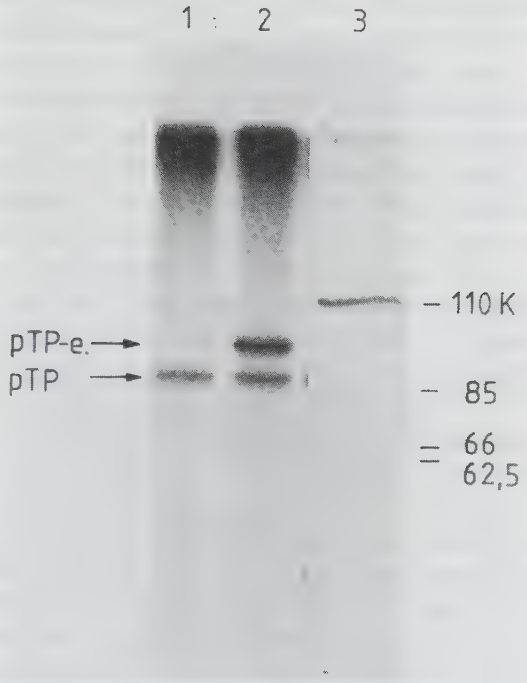
4.3. Initiation of adenovirus DNA replication *in vitro* .

4.3.1. Initiation and limited elongation of Ad2 DNA *in vitro* .

The proposed first step in adenovirus replication is the formation of a complex between the terminal protein precursor (pTP) and the 5'-dCMP residue on the DNA. According to the model, (fig. 3), this complex may then act as primer for chain elongation. Recently, the formation of such a complex was demonstrated *in vitro* by Lichy et al. (1981).

A similar complex is formed using nuclear extracts from Ad2 infected HeLa cells incubated with α-^{32}P-dCTP in the presence of ATP and Ad2 DNA-protein complex as described in 3.11. The product of this test system was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography which revealed a single protein band labelled with ^{32}P migrating slightly slower than the marker adenovirus protein III (figure 6, lane 1). The protein III of adenovirus (penton base) has an estimated molecular weight of 85000 daltons in 10%-polyacrylamide-SDS gels and, based on this value, the complex formed in the assay has an estimated molecular weight of 87000 daltons (87K).

The sequence of Ad2 DNA indicates that the first 25 nucleotides from both 5'-termini contain no guanine residues (Arrand, 1979; Shinagawa, 1979). According to the proposed model, it should be possible to elongate the primer, pTP-dCMP, and then to terminate synthesis at the first dG residue by adding an excess of dideoxyGTP (ddGTP). The expected product would be a deoxyoligonucleotide, 26 nucleotides in length, bound to the precursor terminal protein. When the standard *in vitro* reaction is carried out in the presence of dATP, dTTP (10 - 40µM) and an excess of ddGTP (40µM),



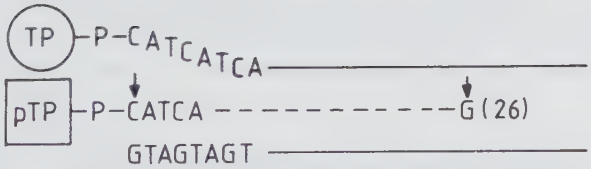


Figure 6. Initiation and limited elongation of Ad2 DNA *in vitro* .

The reactions were carried out as described in 3.11. using Ad2 DNA-protein complex as template. Lane 1: the Ad2 initiation complex; lane 2: limited elongation of Ad2 DNA in the presence of excess ddGTP; lane 3: ^{14}C-labelled Ad2 viral proteins with the apparent molecular weights in kilodaltons (K). The arrow at pTP indicates the position of the Ad2 pTP-dCMP complex and pTP-e. is the elongated form of this complex.

Below: a diagrammatic representation of the Ad2 initiation and elongation events. The arrows indicate respective assay products.

the ^{32}P-labelled pTP band is detected together with a new band which migrates more slowly (figure 6, lane 2). This new labelled complex (approximately 95000 daltons) has been shown to consist of the pTP covalently bound to a nucleic acid moiety of limited length (26 nucleotides) which may be converted to the 87K pTP by complete digestion with micrococcal nuclease (Lichy et al., 1981). Thus, in the elongated product, the nucleic acid "tail" significantly alters the mobility of the precursor terminal protein on a 10% polyacrylamide gel. Even in the presence of dATP, dTTP and ddGTP, the 87000 dalton product is still detected on the gel.
A diagrammatic representation of the Ad2 initiation and elongation events is shown in figure 6.

4.3.2. DNA-protein complex is required for the initiation reaction.

In the absence of exogenously-added DNA-protein template, no pTP-dCMP complex was formed in the *in vitro* initiation assay (figure 7, lane 1). Further, adenovirus DNA treated with proteinase K (3.6.) did not support formation of the initiation complex (fig. 7, lane 2). Partially, deproteinized Ad2 DNA digested with the restriction enzyme Xba I (3.6.1.) was also inactive in the assay (fig. 7, lane 3). Thus, the *in vitro* initiation reaction is apparently dependent on the presence of exogenously-added adenovirus DNA-protein complex as template. The template requirement is not fulfilled endogenously nor by protease-treated DNA.

4.3.3. Stability of the DNA-protein complex.

The adenovirus DNA-protein complex prepared as described in 3.5. was stable in TE/PMSF buffer containing bovine serum albumin (0.5mg/ml) for several months at 4°C. When the Ad2 DNA-protein complex was digested with Xba I for 2h at 37°C and then used, without further treatment, in the *in vitro* reaction, its activity as template in both the initiation and elongation assay was reduced (figure 7, lanes 6 and 7). Undigested Ad2 DNA-protein complex

incubated in a similar fashion with restriction enzyme buffer but without Xba I was also less active in the initiation assay (compare lane 8 with lane 4, fig. 7).

In all experiments using a modified template (i.e. restriction enzyme digested or partially deproteinized) the concentration of DNA was similar to that used in the standard *in vitro* assay with DNA-protein complex as template (approximately 200ng DNA per 25µl assay volume).

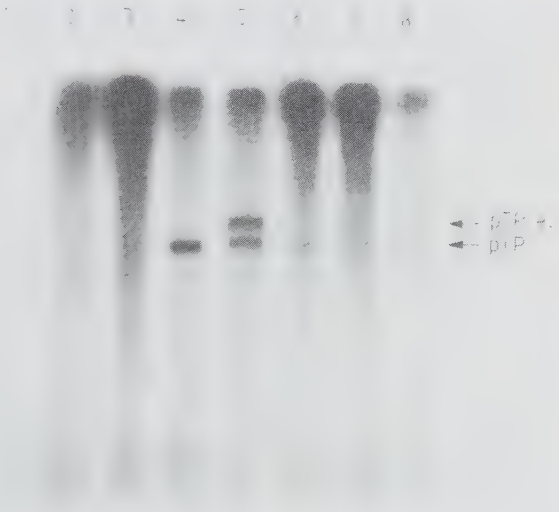


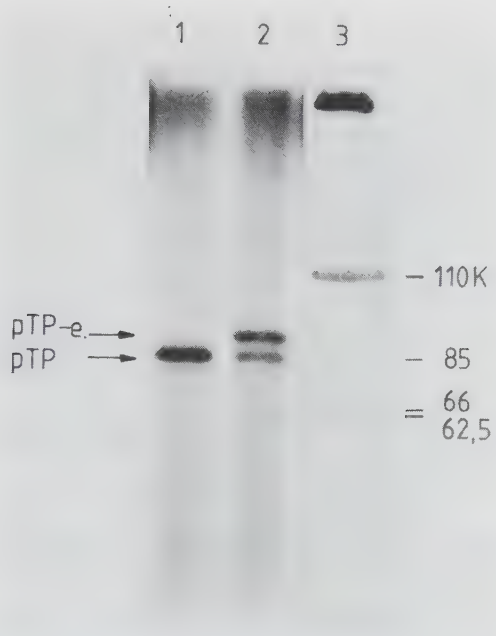
Figure 7. Template requirements for the formation of the Ad2 initiation complex *in vitro*.

The *in vitro* assays for the formation of the initiation complex and limited elongation were performed as described with the following modifications:- lane 1: no DNA; lane 2: protease-treated Ad2 DNA; lane 3: protease-treated Ad2 DNA digested with Xba I; lanes 4 and 5: Ad2 DNA-protein complex; lanes 6 and 7: Ad2 DNA-protein complex digested with Xba I; lane 8: Ad2 DNA-protein complex incubated for 2h at 37°C without enzyme. The positions of the pTP-dCMP complex and its elongated form are indicated.

4.3.4. Initiation and limited elongation of AdFL DNA *in vitro* .

The *in vitro* initiation reaction described in 3.11. was modified slightly in order to investigate early events occurring in the replication of mouse adenovirus strain FL. Nuclei were prepared from AdFL infected mouse 3T3 cells (3.7.2.) and these were extracted with 200mM NaCl on ice for 1 hour. DNA-protein complex was prepared from CsCl-purified mouse virions (3.5.) and served as template for the initiation of AdFL DNA replication *in vitro* . Sequence evidence revealed that the 5'-terminal nucleotide of AdFL DNA was dCMP and that this nucleotide was covalently linked to a protein (Temple et al., 1981). Therefore, α-^{32}P-dCTP could be used, as in the Ad2 system, in an attempt to label the initiation complex. Thus, the *in vitro* assay for AdFL reflected the assay developed for Ad2 replication except that the components were specific for the mouse virus.

The initiation assay described in 3.11. with the modifications discussed above was carried out for 1h at 30°C. The reaction product, after boiling in SDS, was examined on a 10%-polyacrylamide-SDS gel. Autoradiography of the dried gel revealed a single ^{32}P-labelled band migrating somewhat faster than the Ad2 penton base (figure 8, lane 1). Based on the molecular weight estimates for Ad2 structural proteins, the protein labelled in the mouse initiation assay has a molecular weight of approximately 80000 daltons. An attempt to elongate this protein in an assay similar to that used for Ad2 also proved successful. DideoxyGTP was again employed to abruptly terminate chain elongation. Since the first dG residue in the AdFL genome starting from either 5'-terminus occurs at position 19, one would expect to obtain a product consisting of the mouse precursor terminal protein covalently bound to a deoxyoligonucleotide of 19 nucleotides in length. When conditions favouring chain elongation were used in the *in vitro* assay, a second labelled band (approximately 86K) appeared which migrated more slowly than the proposed mouse pTP (figure 8,



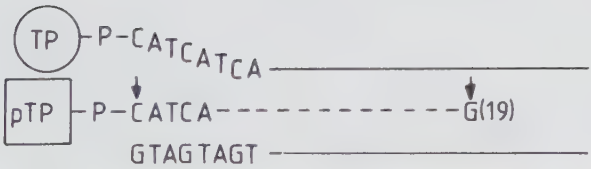


Figure 8. Initiation and limited elongation of AdFL DNA *in vitro*.
The standard *in vitro* assay was performed using mouse AdFL DNA-protein complex as template and nuclear extracts from AdFL infected 3T3 cells. Lane 1: the proposed AdFL initiation complex; lane 2: limited DNA synthesis of AdFL in the presence of excess ddGTP; lane 3: ^{14}C-labelled Ad2 structural proteins in kilodaltons (K). The arrows at pTP and pTP-e. represent the proposed AdFL initiation complex and its elongated form, respectively.

Below: a diagrammatic representation of the AdFL initiation and elongation events. The arrows show the respective reaction products.

lane 2).

Although little evidence is available at present on the nature of the terminal protein linked to AdFL DNA, it was assumed, by drawing an analogy with the Ad2 system, that the 80K labelled band in lane 1 (fig. 8) represents a complex consisting of a precursor terminal protein (mouse pTP) bound to dCMP. Further, it was assumed that the 86K band in lane 2 (fig. 8) represents a mouse pTP covalently linked to a nucleic acid moiety, 19 nucleotides long. The distance between AdFL pTP and its elongated form is smaller than that between the Ad2 pTP and its elongated form (compare fig. 6, lane 2 with fig. 8, lane 2). The size differences between the pTPs of Ad2 and AdFL are discussed further in 5.1.

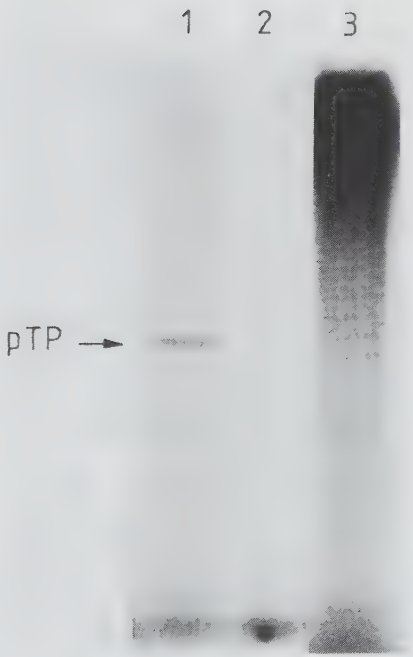
The proposed initiation and elongation events of AdFL replication are shown diagrammatically in figure 8.

4.3.5. DNA-protein complex is required for the AdFL initiation reaction.

If the in vitro initiation assay is carried out in the absence of exogenously-added template, no complex is formed (figure 9, lane 2). Proteinase K treated AdFL DNA is also inactive as template for complex formation (fig. 9, lane 3). Thus, the template requirements are similar to those in the Ad2 system. The initiation reaction appears to be dependent on exogenously-added intact AdFL DNA-protein complex.

Figure 9. Template requirements of the AdFL in vitro system.

The mouse AdFL in vitro assay was carried out as described in the legend to figure 8 with the following modifications:- lane 1: AdFL DNA-protein complex as template; lane 2: no exogenously-added template; lane 3: protease-treated AdFL DNA as template. The arrow indicates the position of the proposed AdFL pTP-dCMP complex.



4.3.6. Initiation on DNA-protein complex templates occurs only in homologous *in vitro* systems.

The initiation and limited elongation assays described for Ad2 and AdFL DNA in 4.3.1. and 4.3.4., respectively, occur in purely homologous replication systems. DNA-protein complex from human Ad2 serves efficiently as template in the *in vitro* assay using nuclear extracts from Ad2 infected human HeLa cells. Likewise, DNA-protein complex from the mouse AdFL is an efficient template in an *in vitro* assay using nuclear extracts from AdFL infected mouse 3T3 cells. The foregoing results also showed that, in both systems, protease-treated DNA could not substitute as template. The

specificity of the nuclear extracts for template was further investigated by determining whether the two functional templates could be exchanged i.e. if DNA-protein complex from either Ad2 or AdFL was active as template in the respective "foreign" replication system. With reference to DNA sequence data, one might expect such interchangeability of templates since the first 17 base pairs of both the Ad2 and AdFL genomes are identical (see figure 1).
Nuclear extracts were prepared from Ad2 infected HeLa cells and AdFL infected 3T3 cells and incubated in the complex formation assay (3.11.) with their natural or "foreign" DNA-protein complexes. As before, the reaction products were analyzed on SDS-polyacrylamide gels followed by autoradiography. The results of these experiments are shown in figure 10. Interchangeability of templates was, however, not found. DNA-protein complex from Ad2 cannot function as template in the "heterologous" in vitro initiation assay i.e. using nuclear extracts from AdFL infected mouse 3T3 cells (fig. 10, lane 1).However, Ad2 template functions efficiently in the initiation and limited elongation assays using the "homologous" in vitro system i.e. nuclear extracts from Ad2 infected HeLa cells (fig. 10, lanes 2 and 3). Likewise, DNA-protein complex from AdFL serves efficiently as template for initiation and elongation in its respective homologous system (fig. 10, lanes 8 and 9), but a "foreign" template i.e. Ad2 DNA-protein complex, is inactive in the mouse in vitro assay (fig. 10, lane 7). The very weak band appearing at 80K in lane 7 upon prolonged film exposure may represent a minimal unspecific complex formation event occurring on some protein-free Ad2 DNA molecules or a "background" initiation event which might be a property solely of the nuclear extracts. However, on comparison with positive initiation events, lane 2 or lane 8, the "activity" seen in lane 7 may be described as negligible (see Discussion).

4.3.7. Nuclear extracts from "mixed" adenovirus/host cell systems retain their template specificity.

Recently, Antoine et al. (1982) have shown that although mouse adenovirus FL grows to only very low titres in human HeLa cells,

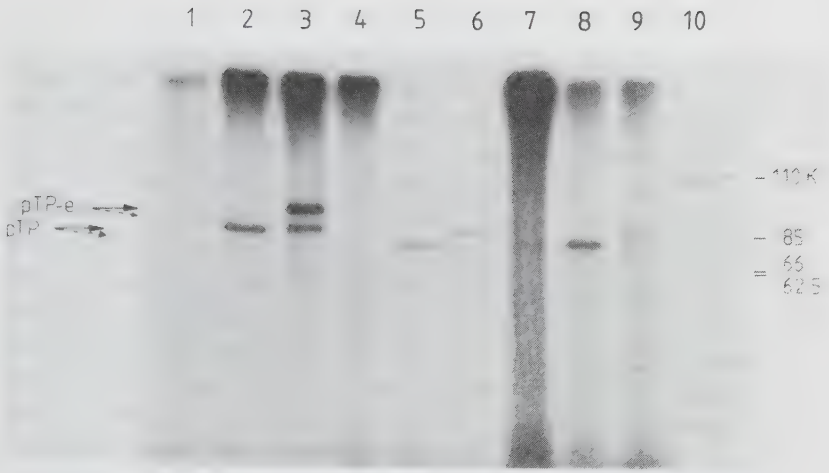


Figure 10. Initiation of adenovirus DNA replication in homologous and heterologous systems.

In vitro assays were performed as described in 3.11. The DNA-protein complexes from Ad2 and AdFL were interchanged and their activity was tested in homologous and heterologous systems (see 4.3.6. and 4.3.7.). Lanes 1-3: nuclear extracts form Ad2/HeLa cells; lanes 4-6: nuclear extracts from AdFL/HeLa cells; lanes 7-9: nuclear extracts from AdFL/3T3 cells. In vitro templates were as follows: lanes 2,3,4 and 7: Ad2 DNA-protein complex; lanes 1,5,6,8 and 9: AdFL DNA-protein complex. ^{14}C-labelled Ad2 virus is shown in lane 10 with the apparent molecular weights of the proteins in kilodaltons (K). The arrows indicate the positions of the Ad2 initiation complex and its elongated form while the broken arrows indicate the proposed AdFL initiation complex and its elongated form.

the rate and extent of AdFL DNA synthesis in HeLa cells is comparable to that in the truly permissive mouse 3T3 cells (see 1.6.). Thus, the synthesis of early viral proteins and viral induced proteins necessary for successful AdFL DNA replication proceeds apparently unimpaired in infected HeLa cells. An advantage of this "mixed" virus/host cell system is that HeLa cells, in contrast to mouse 3T3 cells, can be grown in suspension culture and thus, comparatively large amounts of infected cell nuclei may be obtained.

Nuclear extracts prepared from AdFL infected HeLa cells (3.7.3.)

were incubated with DNA-protein complex from Ad2 or AdFL in the *in vitro* initiation assay as described in 3.11. The results shown in figure 10 reveal that only the mouse AdFL DNA-protein complex serves as template in the initiation assay using the "mixed" AdFL/HeLa nuclear extracts (lane 5). Complex formation does not occur when Ad2 DNA-protein complex is substituted as exogenously-added template (lane 4). Further, when assay conditions are adjusted to favour limited elongation to the first dG residue in the presence of the AdFL template, a second band appears on the gel which is identical in size to the elongated AdFL initiation complex formed by nuclear extracts from AdFL infected 3T3 cells (compare lane 6 with lane 9, fig. 10). Thus, despite the presence of human cell factors, the mouse adenovirus early proteins and viral induced proteins present in these "mixed" nuclear extracts, retain their specificity for the mouse DNA-protein complex as template.

4.3.8. Homologous versus heterologous systems using natural templates.

The foregoing experiments allow a comparison of homologous and heterologous *in vitro* replication systems. The results show convincingly that natural DNA-protein complexes from either Ad2 or AdFL are active templates only in their respective homologous replication assays. The nuclear extracts from adenovirus infected cells can apparently precisely recognize the correct protein-bound DNA terminus and/or specific sequences in that terminus to allow initiation of viral replication to occur in the homologous, but not in the heterologous, *in vitro* assays. This specificity of the nuclear extracts for the template is faithfully conserved even in "mixed" extracts i.e. nuclear extracts from AdFL infected human HeLa cells.
The results are summarized in table 1.

Homologous vs. Heterologous Systems

NE / DNA-P	Ad 2 / HeLa	AdFL / HeLa	AdFL / 3T3
Ad 2	+	-	-
AdFL	-	+	+

Table 1. The DNA-protein complexes of Ad2 and AdFL were tested for template activity in the homologous and heterologous in vitro assay systems. Efficient template activity is indicated as +.
NE = nuclear extract; DNA-P = DNA-protein complex.

4.3.9. Estimation of the molecular weight of the adenovirus precursor terminal protein.

On the basis of their mobilities in SDS-polyacrylamide gels, it was possible to estimate the molecular weights of the precursor terminal proteins of Ad2 and AdFL. The gels were 10% polyacrylamide (polyacrylamide : bisacrylamide, 30 : 0.4) and the molecular weight markers were the structural proteins of adenovirus 2 which were labelled in vivo with ^{14}C-formic acid (3.3.1.). The mobilities of the proteins in the gel were plotted as a function of the logarithm of their molecular weights. The graph (figure 11) indicates an apparent molecular weight of 87000 daltons for Ad2 pTP and 80000 daltons for AdFL pTP.

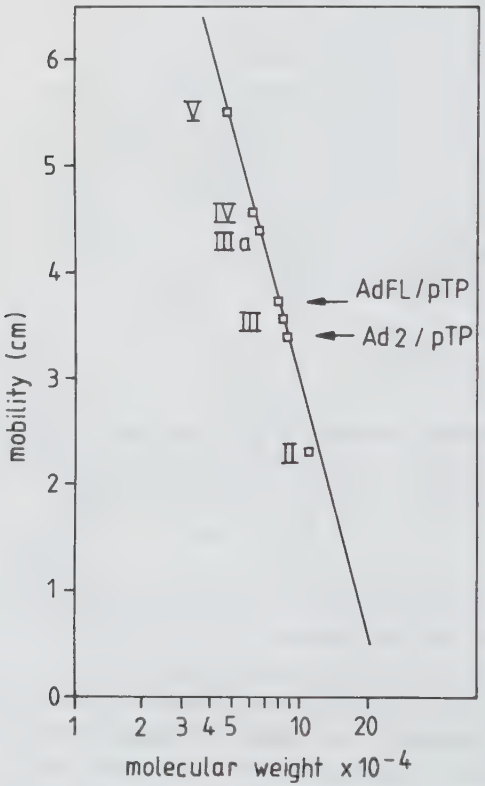


Figure 11. Estimation of the molecular weights of adenovirus pTPs.

The mobilities (in cm) of Ad2 marker proteins were plotted as a function of their molecular weights. The logarithm of the molecular weight was used to obtain a linear representation. Apparent molecular weights for the marker proteins in kilodaltons (K): II(hexon) 110K; III(penton base) 85K; IIIa(vertex region) 66K; IV(fiber) 62.5K; V(core protein) 48K. The molecular weights of the Ad2 and AdFL pTPS from this graph are approx. 87K and 80K respectively.

4.4. Role of nuclear and cytoplasmic factors in adenovirus DNA replication.

The original *in vitro* adenovirus replication assay developed by Challberg and Kelly (1979a) requires a low-salt extract of infected cell nuclei as enzymatic source. These nuclear extracts efficiently supply the components necessary for Ad2 DNA synthesis *in vitro* in the presence of exogenously-added DNA-protein complex, the four dNTPs, ATP and Mg^{2+}. The authors demonstrated that nuclear extracts from uninfected cells were unable to support DNA replication. Further, in the presence of nuclear extracts from infected cells, DNA synthesis was not significantly enhanced by the addition of a cytoplasmic extract from infected cells. The complex formation assay (Lichy et al., 1981) which monitors the initiation event, requires a combination of cytoplasmic extracts from infected cells and nuclear extracts from either infected or uninfected cells. The initiation and limited elongation assays described in 4.3.1. and 4.3.4. rely solely on infected cell nuclear extracts for enzymatic activity. In order to investigate the role of nuclear and cytoplasmic factors in this assay system, nuclear extracts were prepared from HeLa or 3T3 cells exactly as described in 3.7. and 3.8. but without the addition of virus. Cytoplasmic extracts were prepared from infected and uninfected cells as described in 3.8.2.

4.4.1. Host cell nuclear factors are necessary for efficient *in vitro* initiation of Ad2 DNA replication.

The *in vitro* initiation assay described in 3.11. was carried out in the presence of Ad2 DNA-protein complex with the addition of various combinations of nuclear and cytoplasmic extracts from both Ad2 infected and uninfected HeLa cells. In the experiments using a combination of cytoplasmic and nuclear extracts, one volume of cytoplasmic extract (i.e. 3µl) was supplemented with 2.5 volumes of nuclear extract (i.e. 7.5µl) per 25µl assay volume. This ratio was based on the observation that a minimum level of nuclear components

must be present in these combined extracts before successful formation of the initiation complex was possible. Increasing the volume of cytoplasmic extract added did not affect extract activity but drastically increased the background on the gel.

The results of these experiments, shown in figure 12, may be summarized as follows;

- when nuclear extracts alone are used in the in vitro assay, complex formation occurs only when these extracts are obtained from Ad2 infected HeLa cells (compare lane 1 with lane 6).

- if nuclear extracts from infected cells are supplemented with cytoplasmic extracts from uninfected cells, no significant increase in complex formation is observed (compare lane 1 with lane 2). When the cytoplasmic extracts are replaced with cytoplasmic extracts from infected cells, a slight increase in activity is found (lane 3).

- cytoplasmic extracts from infected cells alone possess only a very weak complex formation activity (lane 4). This activity can be greatly stimulated by the addition of nuclear extract from uninfected cells (lane 8).

- cytoplasmic extracts from uninfected cells alone or in combination with nuclear extracts from uninfected cells are completely inactive (lanes 5 and 7 respectively).

- finally, complex formation with cytoplasmic extracts from Ad2 infected HeLa cells may also be stimulated , albeit to a lesser extent, by "foreign" nuclear extracts i.e. nuclear extracts from uninfected mouse 3T3 cells (lane 9).

In summary, the efficient in vitro formation of the adenovirus initiation complex is dependent on viral proteins and viral induced proteins and on one or more host cell factors. The activity of

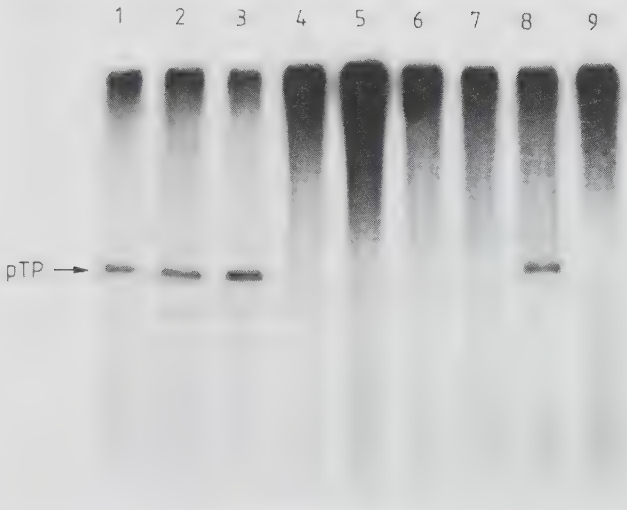


Figure 12. Influence of nuclear and cytoplasmic fractions in adenovirus DNA replication.

The activities of nuclear and cytoplasmic extracts, alone or combined, were tested in the Ad2 *in vitro* initiation assay. NE = nuclear extract from HeLa cells; NE+ = nuclear extract from Ad2 infected HeLa cells; CE = cytoplasmic extract from HeLa cells; CE+ = cytoplasmic extract from Ad2 infected HeLa cells. The addition of extracts was as follows: lane 1: NE+; lane 2: NE+, CE; lane 3: NE+, CE+; lane 4: CE+; lane 5: CE; lane 6: NE; lane 7: NE, CE; lane 8: NE, CE+; lane 9: NE (from mouse 3T3 cells), CE+. The arrow indicates the position on the gel of the Ad2 initiation complex.

cytoplasmic extracts from infected cells alone is almost negligible even when the amount of extract added is increased and the films are exposed for a longer period of time. On addition of nuclear extracts from uninfected cells, the complex formation activity is

greatly stimulated and is comparable with the activity found in infected cell nuclear extracts. Further, these host cell components must not necessarily come from human cells; uninfected mouse 3T3 cells can also complement with nuclear factors.
The results are shown in table 2.

Activities of NE and CE from Ad2 infected and uninfected cells

NE \ CE	no addition	not infected	infected
no addition	–	–	+
not infected	–	–	++
infected	++	++	+++

Table 2. The results of the *in vitro* Ad2 initiation assays using various combinations of nuclear and cytoplasmic extracts from uninfected and Ad2 infected HeLa cells are shown. A complex formation event is indicated as +.
NE = nuclear extract; CE = cytoplasmic extract.

4.5. Initiation of adenovirus replication on protein-free DNA templates.

The results obtained so far are based on experiments employing "natural" viral templates i.e. intact DNA-protein complex isolated as described in section 3.5. from purified Ad2 or AdFL virions. When partially deproteinized DNA (either Ad2 or AdFL) is used as exogenously-added template, no initiation complex is detected within the boundaries of the *in vitro* assay for the human and mouse viruses. Thus, it is tempting to suggest that the presence of the terminal protein covalently bound to the DNA in these natural templates is essential for efficient template activity. Further, the experiments described in sections 4.3.6. and 4.3.7. revealed that the DNA-protein complexes from human Ad2 and mouse AdFL were not interchangeable. Nuclear extracts from infected HeLa or 3T3 cells could specifically distinguish between a homologous and a heterologous template despite the fact that these two templates must appear very similar to the replication enzymes. In both cases, the *in vitro* matrix is a linear double-stranded DNA molecule of comparable length and it possesses a protein covalently bound to both 5'-termini. As noted in the Introduction (1.6.), the genomes of Ad2 and AdFL share little homology. However, sequence analysis of the inverted terminal repetitions of Ad2 and AdFL has shown that the terminal 17 base pairs are common to both viruses (Temple et al., 1981). Since initiation of DNA replication takes place at the termini of the DNA molecule, one might postulate that this short conserved terminal region plays a role in the initiation of DNA replication. In fact, the 17bp region might suffice as a recognition sequence for replication enzymes and thus allow the formation of the initiation complex under suitable *in vitro* conditions. The lack of interchangeability of template between the Ad2 and AdFL *in vitro* assays would argue against this latter hypothesis although it must be remembered that the two DNA-protein complexes also have a different terminal protein (see 4.3.9.). Thus, it is possible that the terminal proteins of Ad2 and AdFL which are different, at least in size, prevent the complex

formation event when the DNA-protein complex is used as template in a heterologous system.

With respect to the results obtained so far, two related questions may be asked:

1. Is the presence of the intact terminal protein covalently bound to the DNA in the DNA-protein complex essential for template activity in vitro ?

2. Since nuclear extracts from adenovirus infected cells can distinguish between two comparatively similar templates, is the recognition signal for successful initiation a certain protein-bound DNA terminus or a specific sequence within that terminus or both ?

In an attempt to answer these questions, it is essential to have protein-free Ad2 and AdFL genomes as well as intact DNA-protein complexes from both viruses. Proteinase K-treated DNA from Ad2 or AdFL prepared as described in 3.6. is, however, not acceptable since even after extensive treatment with the protease, residual amino acids probably still remain attached to the 5'-termini (Carusi, 1977). Therefore, plasmids were employed containing the terminal fragments of the Ad2 and AdFL genomes or short synthetic segments of the Ad2 terminus.

4.5.1. Plasmids containing adenovirus terminal sequences.

The recombinant plasmids described below, pComaI, pGA613 and pTD17, pTD38 were provided by W. Gröger, G. Antoine and T. Dörper respectively. The plasmids pUC8 (approx. 2700bp; Viera and Messing, 1982) and pBR327 (3273bp; Soberon et al., 1980) were used as cloning vectors and served as controls without adenovirus sequences. Since the control and recombinant plasmids possess a functional ß-lactamase gene, the various bacterial stocks could be grown in L-broth containing ampicillin (50µg/ml) as described in

3.12. Plasmid DNA was isolated as described in 3.12.1. and after digestion with restriction enzymes (3.6.1.), the linearized plasmids were examined on agarose gels before being used in the *in vitro* assay (3.9.2.).

pComaI : this recombinant plasmid contains the right-hand terminal Hind III K fragment of Ad2 cloned into pUC8. The 1008bp long K fragment (97.2 - 100%) was isolated from partially deproteinized Ad2 DNA digested with the restriction enzyme Hind III and the remaining peptide residues were removed by alkali treatment. The strands were reannealed and the adenovirus fragment was cloned into a modified pUC8 vector containing a filled-in Eco RI site and a normal Hind III site. The plasmid may be digested with Eco RI and the resulting linear molecule contains an Ad2 specific terminus with a 5'-overhanging Eco RI sequence at one end of the DNA. The structure of pComaI was confirmed by DNA sequencing (W. Gröger, pers. comm.).

pGA613 : this plasmid contains the right-hand terminal Pst I H fragment of the mouse AdFL genome (94.1 - 100%; approx. 1820bp). The terminal fragment was prepared in a similar manner to the Hind III K fragment of Ad2 but was cloned into a pUC8 vector at the Sma I (blunt end) and Pst I sites. The plasmid may be linearized with Eco RI and yields a mouse AdFL specific terminus with a 5'-overhanging Eco RI sequence plus three additional G/C base pairs. The structure of the plasmid was confirmed by DNA sequencing (G. Antoine, Inaugural-Dissertation, München, 1984).

pTD17, pTD38 : these recombinant plasmids contain the terminal 17 or 38 base pairs of Ad2 respectively. The short oligonucleotide sequences were chemically synthesized as described by Dörper and Winnacker (1983) and the fragments were flanked by Eco RI and Hind III 5'-overhanging sequences. These "synthetic termini" were then cloned into the vector pBR327 at the Eco RI and Hind III restriction sites. Plasmid structure was confirmed by DNA sequencing (T. Dörper, pers. comm.).

The structures of all four plasmids are shown in figure 13 and the DNA sequences at the ends of the molecules after linearization with the restriction enzyme Eco RI are also displayed. Typical agarose gels are also shown in figure 14 with both undigested and Eco RI linearized plasmids.

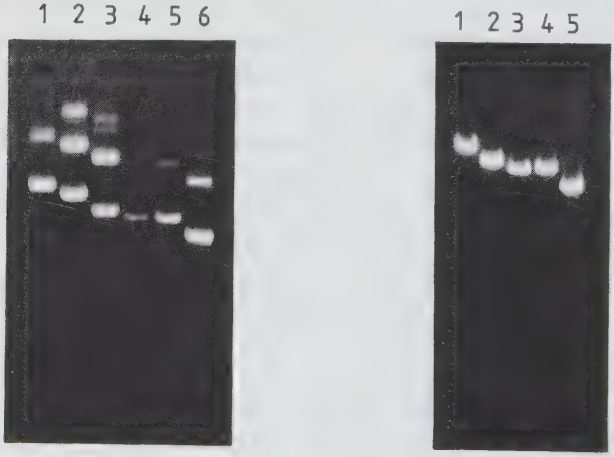
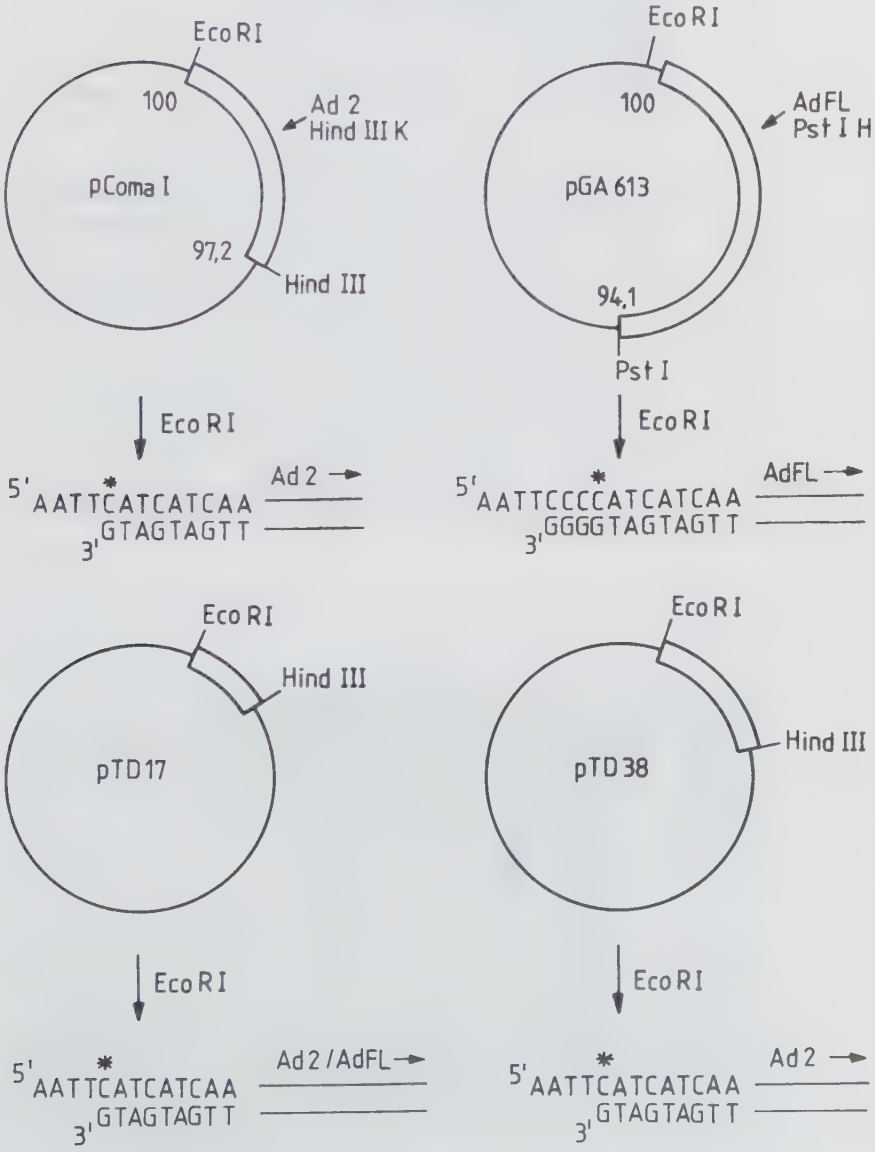


Figure 14. Agarose gel electrophoresis of Ad origin-containing plasmids and vector plasmids.
Left, supercoiled plasmids: lane 1: pGA1127; lane 2: pGA613; lane 3: pComaI; lane 4: pTD38; lane 5: pTD17; lane 6: pUC8. Right, Eco RI linearized plasmids: lane 1: pGA613; lane 2: pComaI; lane 3: pTD38; lane 4: pTD17; lane 5: pUC8. The plasmid pGA1127 contains the left-hand terminus of AdFL (see Antoine, 1984). The other plasmids are described in the text.

Figure 13. Structures of adenovirus origin-containing plasmids.

The structure of the plasmids pComaI, pGA613, pTD17 and pTD38 are shown. Adenovirus-specific sequences are shown by open-boxed regions and relevant restriction enzyme sites are indicated. Ad genome positions (in map units) are shown for Ad2 and AdFL fragments. The DNA sequences after linearization with Eco RI are also indicated under each plasmid and the first Ad nucleotide is marked with *. The plasmids are not drawn to scale.

Plasmids containing origins of adenovirus DNA



4.5.2. Cloned termini of Ad2 DNA support the formation of the initiation complex.

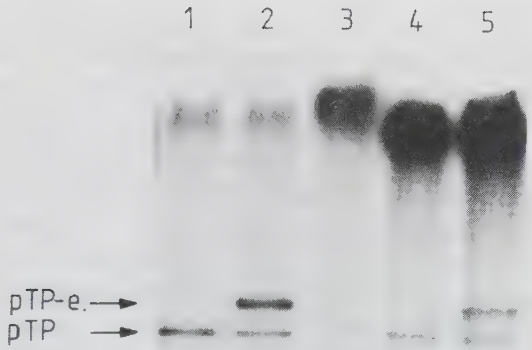
The plasmid, pComaI, containing the right-hand terminus of the Ad2 genome was linearized with Eco RI as described in 3.6.1. The linearized plasmid was then used as exogenously-added template in the Ad2 initiation and elongation assays. As controls, separate experiments were carried out simultaneously using the intact Ad2 DNA-protein complex and undigested, supercoiled DNA from pComaI. The results are shown in figure 15. Linearized DNA from pComaI supports formation of the Ad2 initiation comlex (pTP-dCMP) and allows limited DNA synthesis in the elongation assay (lanes 4 and 5 respectively). These results are identical with those found for the natural template i.e. intact DNA-protein complex (lanes 1 and 2 respectively). Undigested pComaI cannot serve as template in the in vitro assay system despite the presence of Ad2 sequences in the circularized plasmid (lane 3). The concentration of DNA in the assays using pComaI, digested or undigested, was approximately 1µg per 25µl assay volume as compared to approx. 200ng Ad2 DNA-protein complex per assay volume.

Figure 15. Initiation of Ad2 replication on protein-free termini.

Initiation and limited elongation of DNA synthesis were performed using the standard Ad2 in vitro assay and the following templates: lanes 1 and 2: Ad2 DNA-protein complex; lane 3: supercoiled pComaI; lanes 4 and 5: pComaI linearized with Eco RI. The arrows indicate the Ad2 initiation complex and its elongated form.

Figure 16. Template specificity of the Ad2 initiation assay.

The Ad2 initiation and limited elongation in vitro assays were carried out as described using the following DNAs as templates: lane 1: no DNA; lanes 2 and 3: DNA-protein complex; lanes 4 and 5: linearized pTD38; lane 6: linearized pTD17; lane 7: linearized pBR327; lane 8: linearized pUC8; lanes 9 and 10: linearized pGA613. Limited elongation was performed with the samples in lanes 3 and 5 while all other samples were tested only in the complex formation reaction.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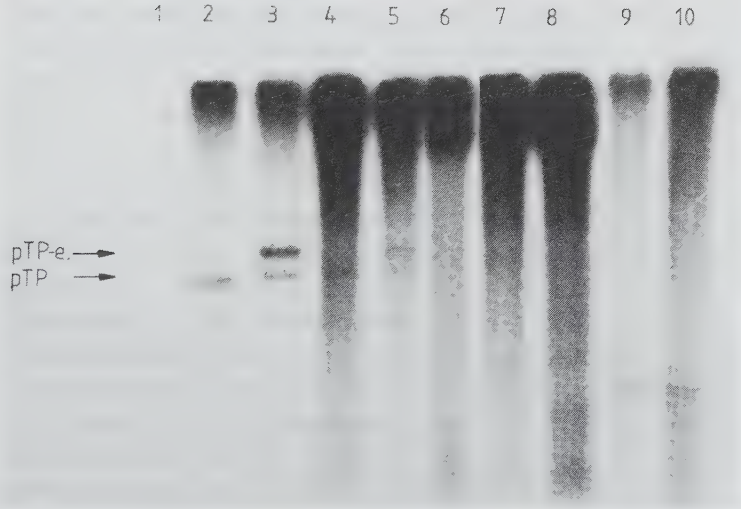
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Similar experiments were then performed using linearized forms of the following plasmids: pTD17, pTD38, pGA613, pUC8 and pBR327. The plasmid pTD38 containing the first 38bp of the Ad2 genome supported pTP-dCMP formation and limited elongation in the *in vitro* system using nuclear extracts from Ad2 infected HeLa cells (figure 16, lanes 4 and 5 respectively). However, all other linearized plasmids showed no activity. The plasmid pTD17 containing the first 17bp of Ad2 DNA was inactive in the initiation assay despite an 80-fold increase in molar DNA termini (fig. 16, lane 6). In a further attempt to initiate the plasmid, pTD17, partially purified extracts from Ad2 infected HeLa cells were used (3.8.1.). However, no *in vitro* complex formation was detected despite high levels of pTD17 DNA and concentrated nuclear extracts (not shown). Linearized DNA from the vector plasmids i.e. pUC8 and pBR327 without any Ad2 inserts could not serve as template in the *in vitro* initiation assay (fig. 16, lanes 7 and 8). Finally, the plasmid pGA613, containing the mouse AdFL terminus, was equally inactive in the assay using nuclear extracts from Ad2 infected HeLa cells (lane 9 and 10).

In summary, nuclear extracts from Ad2 infected HeLa cells can initiate and elongate adenovirus DNA completely freed of the terminal protein. Template requirements are fulfilled by specific adenovirus 2 origins which are longer than the terminal 17 base pairs.

4.5.3. Cloned termini of AdFL DNA also support formation of the initiation complex *in vitro*.

The linearized plasmid pGAb13 represents a protein-free terminus of AdFL DNA. When this linearized plasmid is added as template to the *in vitro* AdFL initiation assay, a labelled band is found on the gel which appears identical to the proposed mouse pTP-dCMP complex. The cloned terminus may also be elongated *in vitro* and a second slower moving band is then found which corresponds to the proposed

elongated AdFL initiation complex (figure 17, lanes 4 and 5 respectively). Therefore, in analogy with the human system, nuclear extracts from AdFL infected mouse 3T3 cells can efficiently initiate and elongate protein-free genome termini. However, by comparing the results shown in figure 15 and figure 17, it is evident that the in vitro inititation event appears to be less efficient with the cloned AdFL terminus than with the cloned Ad2 terminus. This may reflect the activity of the nuclear extracts, although many different infected mouse cell nuclear extracts gave similar results. Alternatively, linearized pGA613 may be less active as template DNA than pComaI since the cloned AdFL terminus has an Eco RI 5'-overhanging sequence plus 3 extra G/C pairs, whereas pComaI has only the Eco RI sequence (see fig. 13). In general, the concentration of linearized pGA613 DNA was approximately 1µg per 25µl assay volume i.e. about a 15-fold increase in molar AdFL termini as compared to the assay using the natural template. Increasing the concentration of linearized plasmid DNA did not significantly alter the intensity of the 80K band.

Initiation and elongation tests were then performed using nuclear extracts from AdFL infected 3T3 cells and linearized forms of the various plasmids described in 4.5.1. as exogenously-added template. The linearized plasmids pComaI, pTD17 and pTD38 all support formation of the initiation complex in vitro . However, all attempts to elongate this complex in the presence of dCTP, dATP, dTTP and excess ddGTP failed. In some cases, the elongation assay allowed formation of the initiation complex (figure 17), while in others, no band was detected at either the 80K or 86K positions on the gel. Finally, when linearized pUC8 or pBR327 DNA were used as template, a weak band was detected at the AdFL pTP-dCMP position. Again, this "initiation complex" could not be elongated under favourable in vitro conditions.

In summary, nuclear extracts from AdFL infected 3T3 cells can successfully initiate and elongate a protein-free AdFL terminus. The weaker in vitro template activity of pGA613 as compared to AdFL DNA-protein complex may reflect the somewhat "hidden" nature of the AdFL terminus in the linearized plasmid due to the

additional nucleotides present at the end of the molecule. The nuclear extracts could also initiate the linearized plasmids pComaI, pTD17 and pTD38 albeit to a lesser extent than pGA613. Despite an apparently positive initiation event, limited elongation to the first dG residue using these plasmids as template was never detected. The control plasmids, pUC8 and pBR327, could also weakly support complex formation with some nuclear extract preparations. However, as with the plasmids pComaI, pTD17 and pTD38, no elongation could be detected with linearized pUC8 or pBR327.
These results seem to indicate a lesser template specificity of the nuclear extracts from AdFL infected 3T3 cells and they are discussed further in section 5.1.

4.5.4. Cloned termini of AdFL DNA support complex formation using "mixed" extracts from adenovirus infected cells.

The "mixed" nuclear extracts obtained from AdFL infected HeLa cells have a specific template requirement; only DNA-protein complex from mouse AdFL supports initiation and chain elongation whereas the Ad2 DNA-protein complex is inactive (see 4.3.7.). Using protein-free templates, the specificity of these extracts was further characterized.
The linearized pGA613 DNA can support in vitro initiation and limited elongation using nuclear extracts from AdFL infected HeLa cells (figure 18). The labelled bands corresponding to the mouse pTP-dCMP complex and its elongated form are again less evident than those obtained using a natural AdFL template (compare lanes 2 and 3 with lanes 4 and 5). The linearized plasmids pTD17, pTD38 and pUC8 could support neither initiation nor limited elongation in the in vitro assay (lanes 6 to 10), even with increased DNA concentrations. Supercoiled and linearized forms of the plasmid pComaI were also inactive as template in both assays as was the linearized DNA of pBR327 (figure 19, lanes 1 to 4). Finally, when partially purified nuclear extracts from AdFL infected HeLa cells (3.8.1.) were used, only linearized pGA613 and the AdFL DNA-protein complex could serve as template in the in vitro assay (not shown).

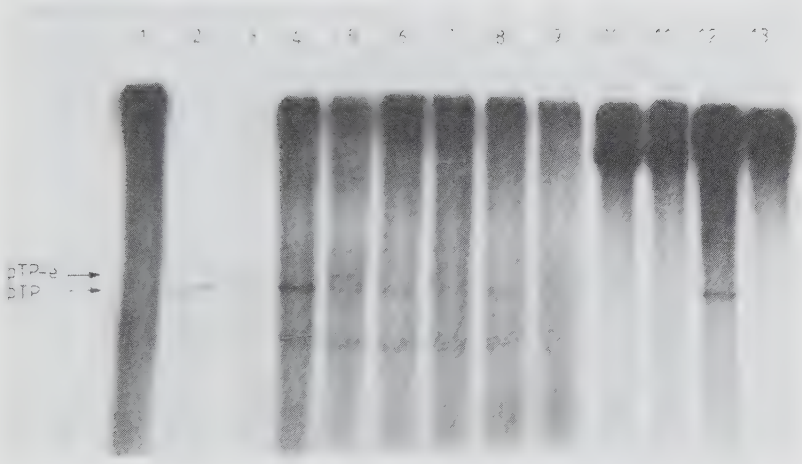


Figure 17. Protein-free templates in the AdFL *in vitro* initiation and elongation assay systems.

Template specificity of the mouse AdFL *in vitro* system was tested using the following linearized DNAs: lane 1: pBR327; lanes 2 and 3: AdFL DNA-protein complex; lanes 4 and 5: pGA613; lanes 6 and 7: pTD38; lanes 8 and 9: pTD17; lane 10: pUC8 linearized with Eco RI; lane 11: pUC8 linearized with Hind III; lanes 12 and 13: pComaI. The samples in lanes 3,5,7,9, and 13 were tested in the elongation assay while all other samples were tested only in the complex formation assay. The arrows indicate the positions of the proposed AdFL initiation complex and its elongated form.

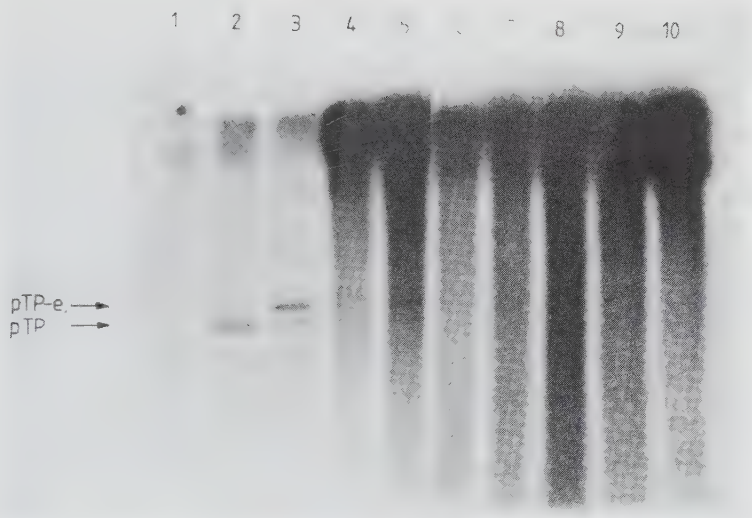
Thus, the specificity of these "mixed" extracts for a mouse viral template is retained when protein-free DNA is employed. Human adenovirus termini i.e. pComaI and pTD38 and very short AdFL termini i.e. pTD17 are unable to function as replication templates *in vitro*. The plasmids pUC8 and pBR327 without any adenovirus sequences are also inactive *in vitro* .

Figure 18. Template specificity of nuclear extracts from AdFL infected HeLa cells.

The "mixed" nuclear extracts (i.e. AdFL/HeLa) were assayed *in vitro* with the following linearized DNAs as templates: lane 1: no DNA; lanes 2 and 3: DNA-protein complex from AdFL; lanes 4 and 5: pGA613; lanes 6 and 7; pTD17; lanes 8 and 9; pTD38; lane 10: pUC8. The initiation reaction was performed with samples 1,2,4,6,8 and 10 and the limited elongation assay was used for samples 3,5,7 and 9. The AdFL pTP-dCMP complex and its elongated form are indicated

Figure 19. Template specificity of AdFL/HeLa nuclear extracts.

The template requirements of nuclear extracts from AdFL infected HeLa cells were investigated *in vitro* with the following linearized DNAs: lane 1: pBR327; lane 2: pComaI (supercoiled DNA); lanes 3 and 4: pComaI (initiation and limited elongation assays respectively); lanes 5 and 6: AdFL DNA-protein complex (initiation and elongation assays respectively). The arrows show the positions of the AdFL pTP and its elongated form, pTP-e.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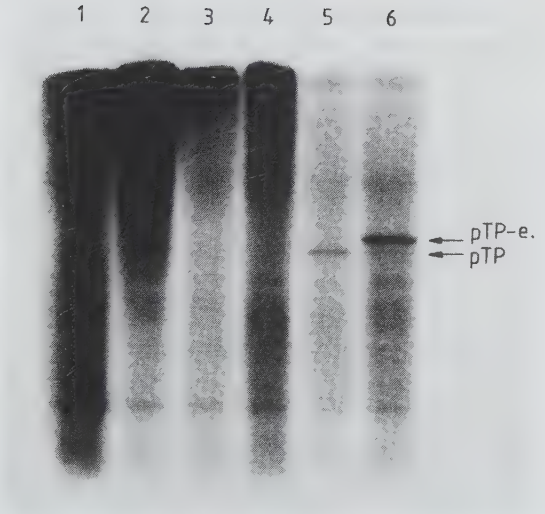
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5. DISCUSSION .

A model system for the study of eucaryotic DNA replication is only really useful if it can be defined by a limited set of components and then reconstructed *in vitro* without loss of specificity. The development of a soluble enzyme system from the nuclei of adenovirus infected cells capable of replicating exogenously-added adenovirus DNA *in vitro* (Challberg and Kelly, 1979a and b) was a major breakthrough in the research on eucaryotic DNA replication. Considerable experimental evidence obtained by various researchers supports the conclusion that DNA replication in this *in vitro* system closely resembles adenovirus DNA replication *in vivo* . The cell free system for the replication of adenovirus DNA has allowed a characterization of the cellular and viral proteins required for viral DNA replication as well as the identification of DNA template requirements in the *in vitro* system.
The experiments described in the preceding sections are concerned with the initial events occurring when replication enzymes recognize an adenovirus DNA molecule as template for DNA synthesis. These initiation events occur at the extreme termini of the adenovirus linear genome (see reviews by Winnacker, 1978; Challberg and Kelly, 1982; Fütterer and Winnacker, 1984) and thus, the experiments are related to the structure of adenovirus termini capable of supporting initiation of DNA synthesis. Further, the experiments are concerned with the role of cellular and viral factors in the initiation of adenovirus replication.

5.1. The structure of a functional template.

The novel mechanism for the initiation of adenovirus DNA replication originally proposed by Rekosh et al. (1977) involves the priming of DNA chain elongation by a deoxyribonucleotide covalently attached to the precursor terminal protein (see figure 3). This "protein priming" mechanism has recently found wide support (Challberg et al., 1980; Lichy et al., 1981; Enomoto et

al., 1981; Pincus et al., 1981; Challberg et al., 1982; Tamanoi and Stillman, 1982; Goding and Russell, 1983; van Bergen et al., 1983). A modified form of the *in vitro* replication assay has been developed to monitor the proposed initiation event in adenovirus DNA replication (4.1.1.).

Nuclear extracts from adenovirus 2 infected HeLa cells support formation of the initiation complex (pTP-dCMP) in the presence of Ad2 DNA-protein complex in the assay described in 3.11. The initiation complex, detected as a ^{32}P-labelled band on a polyacrylamide-SDS gel (figure 6, lane 1), has an apparent molecular weight of 87000 daltons when compared to marker proteins (figure 11). In all *in vitro* experiments described here, the molecular weight markers were the ^{14}C-labelled structural proteins of purified Ad2 virions. The apparent molecular weight values (in daltons) of the major viral structural proteins were taken from Philipson, 1983. However, somewhat different values for the Ad2 capsid proteins are also cited in the literature (Maizel et al., 1968; Flint, 1981), so that an absolute size determination by electrophoresis of the pTP-dCMP complex using Ad2 structural proteins as markers is not possible. The size estimates of the Ad2 initiation complex formed *in vitro* vary from 80K to 87K between different research groups. This discrepancy is probably due to the use of different molecular weight markers. Cell-free translation of the mRNA encoding the Ad2 precursor terminal protein resulted in the formation of an 87K protein (Stillman et al., 1981). However, DNA sequence data of the E2B transcription unit for Ad2 pTP (Smart and Stillman, 1982) predict a molecular weight value of only 74.6K for pTP, assuming that the protein is encoded exclusively in this region of the genome (see 1.2.2.). The higher molecular weight value for pTP might thus be achieved via post-transcriptional processing (e.g. RNA splicing) or post-translational modification.

The Ad2 initiation complex may be elongated to the first dG residue by addition of excess ddGTP in the presence of the other three dNTPs. In this reaction a second slower moving band appears on the gel. This labelled band, when eluted from the gel and treated with alkali, migrated as a 26-base oligonucleotide on a polyacrylamide/urea gel (Lichy et al., 1981; Tamanoi and Stillman,

1982). If the elongation reaction mixture is treated with micrococcal nuclease and then applied to the gel, only the pTP band is seen and not its elongated form (Lichy et al., 1981). These results strongly suggest that the slower moving labelled band in figure 6 (lane 2), which is formed under conditions similar to those described by Lichy et al. (1981), consists of the Ad2 precursor terminal protein covalently attached to a 26-base deoxyoligonucleotide. As seen in fig. 6, even under conditions favouring DNA chain elongation, the labelled initiation complex (pTP-dCMP) was still detected on the gel. This result demonstrates the inability of some initiation complexes to elongate possibly because of an unstable association with the template DNA-protein complex. Recent studies on the kinetics of adenovirus DNA replication (Bodnar and Pearson, 1980a and b) have shown that the genomes of several different serotypes are replicated in approximately 20 minutes at 37°C. On the other hand, the doubling time for the production of viral DNA during the logarithmic growth phase is approximately 60 minutes which suggests the existence of a rate limiting step in adenovirus DNA synthesis. This crucial step may well occur at the initiation stage and the failure of some pTP-dCMP complexes to elongate *in vitro* may reflect some rate limiting step in *in vivo* DNA synthesis. Alternatively, the pTP product in the elongation assay may result from rapid degradation of elongated complexes by nucleases in the nuclear extract or some factor, essential for chain elongation, may be present only in a limited supply in the nuclear extracts. Finally, initiation of adenovirus DNA replication is not dependent on further chain elongation since even in the presence of excess ddATP, the pTP-dCMP complex is formed.

Little is known about the terminal protein of the mouse adenovirus FL except that the 5'-termini of the linear DNA are linked to a protein (Larsen and Nathans, 1977; Temple et al., 1981). The size of the AdFL terminal protein and its possible synthesis as a larger precursor are unclear. A molecular weight estimate of the protein from the corresponding coding region on the genome is also impossible since there is, at present, no available transcription map for the AdFL genome. Therefore, the results obtained in figure

8 for the initiation and elongation of AdFL DNA are interpreted on the basis of previous knowledge of the Ad2 *in vitro* assay system. It was assumed, by drawing an analogy with the Ad2 system, that the labelled band in lane 1, fig. 8, represents a mouse AdFL initiation complex and that the upper band in lane 2, fig. 8, is the mouse precursor terminal protein (pTP) covalently linked to a 19-base deoxyoligonucleotide. Both of these assumptions are strongly supported by the fact that the assay conditions and template requirements for initiation and limited elongation are identical in both adenovirus systems except that the AdFL *in vitro* assay requires nuclear extracts from AdFL infected 3T3 cells and DNA-protein complex from AdFL virions as template. Further, the proposed AdFL pTP may be successfully elongated in the limited elongation assay and produces a second slower moving band (approx. 86K) which is not only smaller in size than the elongated form of Ad2 pTP, but also runs closer to the AdFL pTP (compare lanes 3 and 9 in fig. 10). The shorter distance between AdFL pTP and its elongated form is expected since the first dG residue in the AdFL genome occurs at position 19 and thus, a shorter nucleic acid "tail" is attached to a smaller protein in the AdFL system. Therefore, the experimental evidence presented here supports the view that the mouse AdFL DNA is also initiated by a "protein priming" mechanism. This is, perhaps, not too surprising since it has been established that the pattern of AdFL DNA replication is identical to that of Ad2 DNA (Temple, 1980; Temple et al., 1981). The smaller size of the proposed AdFL pTP is in keeping with the overall smaller size of the mouse adenovirus (see 1.6.). However, the true identity of the AdFL pTP formed *in vitro* may only be confirmed when the mature terminal protein of AdFL DNA is better characterized e.g. size determination by iodination of the AdFL DNA-protein complex with ^{125}I *in vitro* using the Bolton and Hunter reagent (Bolton and Hunter, 1973).

The *in vitro* initiation and limited elongation assays for Ad2 and AdFL are template-dependent reactions i.e. no initiation complex is formed in the absence of exogenously-added viral DNA-protein complex. Nuclear extracts with acceptable *in vitro* activities

could be prepared by using rapidly-growing, mycoplasma-free cells infected with virus at relatively high multiplicities of infection (20 - 100pfu/cell). Both assays were reproducibly active in their respective homologous systems i.e. human or mouse assay systems. Since both homologous *in vitro* systems were active, it was decided to exchange templates and examine whether the heterologous *in vitro* systems were equally active. Recently, Green et al. (1979a) showed that the DNAs from adenoviruses 12, 7, 2, 19 and 4, representing Ad groups A - E respectively, all contain covalently bound terminal proteins of about 55000 daltons. The five adenovirus groups show less than 20% homology by DNA hybridization but their respective terminal proteins are, nevertheless, highly related in the primary sequence, as judged by chymotryptic, two-dimensional peptide maps of ^{125}I-labelled proteins. Thus, the viral gene coding for the adenovirus terminal protein must be a highly conserved one, at least among the human serotypes (Rekosh, 1981). Further, the DNA-protein complexes from the five human adenovirus groups (A - E) can act as template for both the initiation and elongation of DNA replication *in vitro* using nuclear extracts from Ad2 infected HeLa cells (Stillman et al., 1982). The heterologous (i.e. from human serotypes other than Ad2) template DNA-protein complexes were all less active than the homologous Ad2 template. The experiments suggested that despite relatively little DNA sequence homology between the various genomes, an apparently highly-conserved terminal protein attached to the heterologous templates might be sufficient to support viral DNA replication, albeit to a lesser extent. However, the results could also be explained by sufficient DNA sequence homology at least at the termini of human adenovirus genomes which in turn would support DNA replication.

The heterologous systems discussed below refer to nuclear extracts from Ad2 infected HeLa cells and AdFL DNA-protein complex as template (i.e. a non-human adenovirus template DNA) or vice versa (see 4.3.6.). The results of these experiments clearly show that interchangeability of templates between the human and mouse adenovirus systems is not permitted: DNA-protein complex from Ad2 or AdFL can function efficiently in the initiation and limited

elongation assays only in their respective homologous systems. Thus, the *in vitro* assay system is highly specific since the replication proteins can carefully detect the correct template. This specificity with respect to template recognition appears to reside solely in the viral components of the nuclear extracts. Because AdFL DNA replicates efficiently in human HeLa cells (Antoine et al., 1982), it was possible to prepare nuclear extracts from AdFL infected HeLa cells. These nuclear extracts could support *in vitro* initiation and limited elongation only with the AdFL DNA-protein complex. Despite the presence of human cell factors, Ad2 DNA-protein complex could not serve as template in this "mixed" adenovirus/host cell system. Thus, it is impossible to replicate Ad2 DNA with non-human viral proteins and, while nuclear extracts from Ad2 infected HeLa cells can replicate DNA from several different human serotypes (see above), they are completely inactive when mouse AdFL DNA is used as exogenously-added template *in vitro* . Yet, both DNA-protein complexes (Ad2 and AdFL) must appear very similar to the DNA replication enzymes. However, the terminal proteins on the Ad2 and AdFL genomes are different in size and probably in sequence (see 1.6.) and this might explain the results in table 1. A closer examination of the DNA terminal sequences of both these and other adenovirus genomes helps to clarify *in vitro* template requirements.

It has been established that the origins of adenovirus DNA replication are at the termini of the linear genome (Ariga and Shimojo, 1977; Sussenbach and Kuijk, 1978a; Reiter et al., 1980). Thus, replication enzymes and other factors of both cellular and viral origin must specifically recognize certain features at the ends of the DNA molecule since internal origins of Ad DNA replication have never been found. As discussed above, the terminal protein, which is found on all adenovirus genomes examined so far, appears to be an essential component of a functional *in vitro* DNA template. This conclusion is based on the observation that protease-treated DNA-protein complex cannot fulfill the template requirements of the *in vitro* replication assay (see 4.3.2. and 4.3.5.). However, protease-treated DNA is apparently not completely

free of peptide residues (Carusi, 1977) and so, the possible role of DNA terminal sequences in template specificity may only be determined through the use of protein-free cloned adenovirus termini. Recently, Tamanoi and Stillman (1982) have shown that nuclear extracts from Ad2 infected HeLa cells, which were partially purified by either denatured DNA cellulose chromatography or by precipitation with 35% ammonium sulphate, could initiate DNA replication *in vitro* using either Ad2 DNA-protein complex or an adenovirus origin-containing plasmid as template. An Ad origin-containing plasmid could only serve as *in vitro* template when it was linearized such that the terminus of the Ad genome was located at the extreme end of the plasmid. Neither undigested, supercoiled plasmid DNA nor linearized molecules with several base pairs preceding the viral terminus were capable of supporting complex formation. Thus, the activity responsible for pTP-dCMP complex formation recognizes both a specific adenovirus sequence and its terminal location on the template DNA.

The plasmid pComaI contains the right-hand terminus of Ad2 (see figure 13). When this plasmid is linearized with Eco RI and used as template DNA, it can efficiently support formation of the initiation complex in the Ad2 *in vitro* assay system (figure 15). Thus, a linear double-stranded DNA molecule with only one adenovirus terminus can replace the "natural" template, at least *in vitro*. Undigested, circular pComaI DNA was completely inactive as template *in vitro*. In general, crude nuclear extracts from Ad2 infected HeLa cells were used to initiate pComaI DNA, although partially purified ammonium sulphate extracts were also active with linearized pComaI DNA as template (not shown). This result is in contrast to those of Tamanoi and Stillman (1982) who found that complex formation with linearized plasmid DNA containing an Ad2 origin was not possible using crude, unfractionated nuclear extracts. They thus suggested the presence of an inhibitory factor, e.g. a nuclease, in crude extracts which might digest the "naked" 5'-termini of the linearized plasmid molecules. However, van Bergen et al. (1983) could also initiate origin-containing plasmids with crude nuclear extracts using DNA concentrations that were at least 5-fold higher than that for DNA-protein complex. A high

concentration of DNA was not absolutely necessary in the experiments described in this work, since even with relatively low plasmid DNA concentrations (300 - 500ng/assay), initiation was possible using active nuclear extracts. Instead, the activity of the nuclear extract used in the individual experiments was crucial. Almost all nuclear extracts were active in supporting initiation of DNA synthesis when the natural DNA-protein complex was used as template. On the other hand, very few preparations could efficiently support initiation on linearized pComaI to the same extent. This result suggests that some factor, essential for the initial event in Ad DNA synthesis, is present in a limited amount in the infected cell nuclear extracts. In the most active extracts, this component must be present at sufficient levels in the crude preparations to successfully recognize pComaI DNA as template. By partially purifying the less active extracts (Tamanoi and Stillman, 1982; this work) this factor might be sufficiently concentrated to permit complex formation on the plasmid DNA template. In experiments using the DNA-protein complex, some feature of this "natural" template, e.g. the terminal protein, might amplify the recognition signal for DNA synthesis, so that the enzymes and other factors required for replication might efficiently recognize a template molecule even when these components are present at very low levels. In conclusion, the parental terminal protein (TP) is apparently dispensable for Ad DNA replication in vitro although a possible role in the more efficient recognition of a "start" signal for DNA synthesis cannot be excluded.

It was also possible to partially elongate the pTP-dCMP complex using linearized pComaI DNA (fig. 15, lane 5). The upper band in lane 5 appears to be a doublet. Both of the authors mentioned above (Tamanoi and Stillman, 1982; van Bergen et al., 1983) obtained similar results and, upon hydrolysis of the elongation products, they found two oligonucleotides corresponding to 23 and 26 bases in length. Thus, the double band seen in lane 5, fig.15, may represent elongated products which are initiated at either the first or second 3'-GTAGT-5' of the 3'-strand of the adenovirus template DNA (see fig.13). This abnormal start might occur due to the presence of the extra sequences at the 5'-end of pComaI (fig.13) or may

reflect the absence of a "site directing" terminal protein.
When the plasmid pTD38, which contains the first 38bp of the Ad2 genome, was linearized with Eco RI, it could also support initiation and limited elongation in the Ad2 in vitro assay system. Supercoiled pTD38 DNA and all other plasmids (pTD17, pGA613, pUC8 and pBR327) were inactive as templates even at high DNA concentrations. Thus, the origin of Ad2 DNA replication must be greater than 17bp in length. The plasmid pGA613 contains a large segment (approx. 1820bp) of the mouse AdFL genome including the entire inverted terminal repetition (93bp in AdFL). Despite the large size of its insert, linearized pGA613 cannot serve as template in the Ad2 in vitro assay presumably because it only shares the terminal 17 base pairs with the Ad2 genome. Therefore, even in the absence of the parental terminal protein, a genome terminus stemming from the mouse AdFL is not a template for human adenovirus replication enzymes. A successful initiation event in vitro requires certain additional sequences beyond position 17 on the Ad2 genome. These sequences are not present in pTD17 and are very different in pGA613 (see figure 1). This short DNA sequence between 17 and 38 base pairs in length defines the minimal in vitro origin of Ad2 DNA replication and has been examined further by other research groups. Tamanoi and Stillman (1983) could show recently that deletions from an internal restriction enzyme cleavage site which retain 20 base pairs or more of the adenovirus terminal sequence in a plasmid could support initiation and limited elongation in vitro . Deletions which leave 14bp or less of the terminus are inactive while all deletions extending from the extreme terminus of the genome inwards also destroy template activity. They, therefore, suggested that the terminal 20 base pairs constitute a functional in vitro origin for the initiation of Ad2 DNA synthesis. Somewhat different results were obtained by van Bergen et al. (1983) using various deletion mutants of plasmids containing Ad 12 terminal fragments. They propose that a certain distance must be maintained between the priming nucleotide site (i.e. dG on the 3'-strand) and the conserved 10bp sequence present in all human adenovirus serotypes between positions 9 and 18 of the genome terminus (see 1.2.1.). Based on their results, they suggest

that any dG residue situated at a distance between 4 and 8 nucleotides from position 9 on the genome can serve as template for Ad DNA synthesis *in vitro*. Thus, in contrast to the results of Tamanoi and Stillman (1983), it appears that certain deletions at the extreme termini of the Ad genome are permitted. Finally, deletion mapping experiments by Enns et al. (1983) also located the adenovirus origin within the first 20bp of the inverted terminal repetition. These latter experiments are unusual in that supercoiled plasmids containing adenovirus terminal fragments are replicated as rolling circles with displaced, single-stranded tails using extracts from Ad2 infected cells (Pearson et al., 1983; Enns et al., 1983). The authors showed that deletions penetrating into the conserved sequence from either direction destroyed the template activity of the cloned adenovirus origins, and therefore proposed that this sequence alone constitutes the signal for the adenovirus origin.

Despite the somewhat different results discussed above which may simply reflect different *in vitro* assay systems, all the experimental evidence to date demonstrates an absolute requirement for the DNA sequence 5'-ATAATATACC-3' between positions 9 and 18 on the genome. As mentioned in the Introduction, this sequence is perfectly conserved in all human Ad serotypes and all or part of this sequence is also present in the ITRs of non-human adenoviruses. It is possible that this A/T-rich region serves as a binding site for the pTP-140K complex (see below) during the initiation of Ad DNA replication. Finally, all these results demonstrate that the origin of Ad DNA relication is indeed very small (i.e. the terminal 20bp of the genome) at least *in vitro*. However, the proposed origins of other procaryotic and eucaryotic genomes characterized to date are comparable in size. For example, a 30bp region is conserved between the single-stranded DNA phages, ØX174, G4 and St-1, and this sequence is believed to represent the genome origin (Heidekamp et al., 1980). Obviously, the question remains as to the function of other sequences at the termini of the genome and, in particular, of the inverted terminal repetition present at both ends of the DNA molecule. There are certain conserved sequences present in the distal G/C-region of the ITRs of

most adenoviruses which are also found in other eucaryotic origins of replication e.g. the human BK virus, simian virus 40 and the mouse polyoma virus (Seif et al., 1979). It is tempting to suggest that such broadly conserved sequences may function as recognition signals for host cell proteins involved in viral DNA replication. Whether these and other terminal sequences of the adenovirus genome are required for efficient in vivo DNA replication remains to be seen.

Before summarizing the features of a functional in vitro template for adenovirus DNA replication, it is necessary to interpret the results obtained in the mouse AdFL in vitro system. The major difficulty with the AdFL/3T3 in vitro assay was the very low yield of nuclei obtained from infected cells. Mouse 3T3 cells, which can only be grown in monolayer cultures, were very sensitive to the relatively high levels of AdFL used for infection and also to the treatment with hydroxyurea. Thus, individual preparations of nuclei from infected cells could not be used in repeating various in vitro experiments and partially purified extracts were not prepared, again due to the initial low yields of nuclei. As in the Ad2 system, most nuclear preparations were active in vitro with the "natural" template i.e. AdFL DNA-protein complex. Some of these extracts could also initiate and elongate linearized pGA613 which contains a terminal fragment of the AdFL genome. Therefore, the parental terminal protein on mouse AdFL DNA is also dispensable for in vitro replication. However, the results in figure 17 indicate that linearized pGA613 is less active as template in the AdFL assay system than linearized pComaI in the Ad2 system (compare fig. 17 with fig. 15). As noted in 4.5.3. this may be due to the somewhat "hidden" nature of the cloned AdFL terminus. Alternatively, the poorer results with a protein-free template may reflect a reduced specific activity of the crude nuclear extracts. Support for this latter explanation comes from the experiments with various linear and supercoiled plasmids containing Ad2 genome origins. Thus, many nuclear extracts from AdFL infected 3T3 cells could apparently initiate linearized pTD38 and pComaI in vitro . These two plasmids have only the terminal 17bp im common with pGA613 which suggests

that a 17bp AdFL sequence is sufficient for DNA replication. However, the labelled band found on the gel with pTD38 or pComaI as template could never be elongated under the standard *in vitro* conditions suitable for limited elongation to the first dG residue. The inability of these templates to support limited DNA synthesis suggests a somewhat unspecific initiation event in AdFL DNA replication *in vitro*. Thus,it is possible that sufficient sequence information is present to allow formation of a pTP-dCMP complex. The absence of further sequences which are apparently necessary for subsequent elongation (e.g. for binding viral or cellular factors required in DNA synthesis) results in the dissociation of the initiation complex from the template without further DNA synthesis. A reduced specificity of these extracts may also explain why complex formation, but not limited elongation, could occasionally be detected when supercoiled plasmid DNA, including the plasmids pUC8 and pBR327, were used as templates *in vitro*. This "unspecific" complex formation event in the AdFL *in vitro* system may in fact be a property solely of the nuclear extracts, since even in the absence of exogenously-added template, a weak band at 80K was seen after prolonged exposure with certain nuclear preparations. Further, a weak 80K band was detected in some cases even with the Ad2 DNA-protein complex as template.

Pearson et al. (1983) observed specific "minor origins" in the plasmid pBR322 which are apparently initiated at low frequencies by extracts from Ad infected cells in an *in vitro* system based on a rolling circle model of replication. Ikeda et al. (1982) recently demonstrated that the circular, single-stranded DNA of the bacteriophage ØX174 could serve as template for the initiation of DNA synthesis at specific genomic sites using a purified fraction from Ad infected cells under certain *in vitro* conditions. The presence of ATP and nuclear extract from host cells were inhibitory in the *in vitro* reaction using single-stranded DNA as template (Ikeda et al., 1982; Tamanoi and Stillman, 1983). Therefore, nuclear extracts from AdFL infected 3T3 cells may be poor in, or lack certain host cell components and thus, occassionally initiate DNA synthesis on various plasmids in a reaction similar to that described for single-stranded DNA. It was not possible to determine

whether initiation on the plasmids discussed above was site-specific since elongation was never detected using the standard nuclear preparations. Finally, linearized pTD17 comprises the first 18bp of the AdFL genome since the Hind III site supplys the dA residue at position 18 on the AdFL genome (see figure 1). The plasmid however cannot support limited elongation in the AdFL assay system despite the presence of an additional nucleotide from the AdFL terminus. This may reflect the activity of the crude nuclear extracts used in the in vitro assay, and protein purification may indeed be necessary for efficient in vitro DNA synthesis (see Tamanoi and Stillman, 1983). Alternatively, the 18bp segment of the AdFL genome may be too short to serve successfully as template for limited AdFL DNA synthesis in vitro.

The results obtained with the nuclear extracts from AdFL infected HeLa cells were, however, more specific. Complex formation in vitro occurred with the AdFL DNA-protein complex and, to a lesser extent, with linearized pGA613 as template. Limited DNA synthesis was also possible with both the "natural" template and the protein-free AdFL terminus. All other plasmids tested and the Ad2 DNA-protein complex were completely inactive in this system. Nuclear extracts which were partially purified with 40% ammonium sulphate could initiate and elongate only the AdFL DNA-protein complex and linearized pGA613 (not shown). The increased specificity of these extracts as compared to those from AdFL infected 3T3 cells might be explained by the presence of a human cell factor which could be more discriminatory than the corresponding mouse cell factor in viral DNA replication. On the other hand, the results could simply reflect increased levels of host cell components. The observation that pTD17 is inactive as template in the AdFL/HeLa system suggests that the 18bp fragment is too short to serve as an AdFL template, at least in the in vitro assay system consisting of mouse viral proteins and human cell proteins.

In summary, a functional *in vitro* adenovirus template appears to have the following structural requirements:

- an adenovirus terminus containing more than the first 17bp of the genome (20bp appear to suffice)

- a linearized DNA molecule with the Ad sequence at or very close to the ends of the molecule (however, see Pearson et al., 1983 for rolling circle model)

- an absolute requirement for the sequence 5'-ATAATATACC-3' within that terminus

- the covalently-bound terminal protein appears to be dispensable for viral DNA replication at least *in vitro* . A role in increasing template efficiency is possible e.g. amplifying the "signal" for recognition by replication enzymes or protection from nuclease digestion.

At present, it not possible to say whether these features of a functional *in vitro* template are identical with those of a viral template supporting DNA replication *in vivo* .

5.2. Host cell components in adenovirus DNA replication

Low-salt nuclear extracts from adenovirus infected cells support the initiation and limited elongation of adenovirus DNA replication *in vitro*. The results in section 4.4.1. show that cytoplasmic extracts from infected cells can also support complex formation *in vitro* when supplemented with nuclear extracts from uninfected cells. The activity of the infected cell cytoplasmic extracts alone was almost negligible. Thus, the efficient initiation of Ad DNA replication requires the presence of one or more host cell nuclear components. This nuclear component can be supplied by other cells which are not normally host cells for Ad2 e.g. nuclear extracts

from mouse 3T3 cells (4.4.1.) or nuclear extracts from hamster CHO cells (Longiaru and Horwitz, 1981). Thus, the cellular component must be a relatively well-conserved one. The dependence of adenovirus DNA replication on host cell nuclear factors is not surprising since the small size of the viral genome limits its coding capacity for replication proteins.

The role of host cell factors in adenovirus DNA replication has recently been the subject of intensive research. So far, two host proteins have been shown to participate in Ad DNA replication. These proteins and viral-coded proteins have been purified and the in vitro replication assay has been reconstituted using purified proteins.

The virus-coded precursor terminal protein, pTP, has already been discussed. The presence of a non-DNA-bound protein has recently been demonstrated in infected cells (Green et al., 1981) and the pTP of Ad2 has also been purified in a functional form (Enomoto et al., 1981). Isolated pTP does not contain a known enzymatic activity but is tightly bound to a 140000 dalton protein which contains a DNA polymerase activity that is different from host cell polymerases (Lichy et al., 1982). This adenovirus DNA polymerase (Adpol) is encoded by viral early region E2B (Stillman et al., 1982b), is directly involved in the formation of the initiation complex at the termini of the adenovirus genome and can use the pTP-dCMP complex as primer for DNA synthesis. Host cell proteins are also required for successful Ad initiation in vitro (see above). Nagata et al. (1982) have recently purified a host cell nuclear protein, nuclear factor I, of apparent molecular weight of 47000 daltons. This cellular factor is required for formation of the pTP-dCMP complex when double-stranded DNA is used as template but may be omitted when single-stranded DNA is used. Nuclear factor I has no apparent enzymatic activity but binds to a specific DNA sequence within the Ad inverted terminal repetition located at positions 17 to 48 of the genome (Nagata et al., 1983b).

Two other proteins involved in Ad DNA replication are also of interest. The single-stranded DNA binding protein DBP (72K) is encoded by viral early region E2A and is involved in elongation of DNA chains but is not required for the initiation reaction (see

Stillman, 1983). Another cellular protein (nuclear factor II) allows completion of Ad DNA replication *in vitro* and has been shown to contain a type I topoisomerase-like activity (Nagata et al., 1983a). Finally, ATP is essential in the *in vitro* initiation and replication assays using double-stranded DNA as template. ATP can reduce dCTP breakdown during incubation and it is possible that the nucleoside triphosphate functions by protecting dCTP rather than having a direct role in the initiation reaction (De Jong et al., 1983). Alternatively, a DNA-dependent ATPase could be required in the initiation reaction (e.g. partial unwinding of the duplex) and ATP may thus serve as substrate for this reaction. Several DNA-dependent ATPases are known to function in the initiation of DNA replication in procaryotic systems e.g. the protein n' has an ATPase activity and is involved in the initiation of ØX174 DNA replication.

Many of the steps involved in the initiation of adenovirus DNA replication have been elucidated. However, several problems still remain e.g. are *in vitro* events identical with those *in vivo* ? In this respect, the replication of the displaced strand has not been reproduced *in vitro* and the possibility of a different initiation mechanism involving other proteins cannot be excluded. The answers to these questions have yet to be determined.

6. SUMMARY .

The initiation of adenovirus DNA replication was investigated in a cell-free system using human Ad2 and mouse AdFL as model systems. According to the "protein priming" model, the initial event is the formation of a covalent complex between the Ad precursor terminal protein, pTP, and the first nucleotide residue of the genome, dCTP, resulting in the initiation complex, pTP-dCMP. Nuclear extracts from Ad2 infected HeLa cells can successfully initiate DNA synthesis *in vitro* using the Ad2 DNA-protein complex as template. Limited elongation to the first dG residue is also possible. During this work, an *in vitro* assay system was developed to examine the initiation of AdFL DNA replication. Nuclear extracts from AdFL infected mouse 3T3 cells can support initiation and limited elongation using DNA-protein complex from AdFL purified virions. The proposed AdFL pTP was shown to be smaller in size (i.e. 80K) than the pTP of Ad2 (i.e. 87K). Despite the reproducibility of these homologous *in vitro* systems (i.e. Ad2 with human cell extracts and AdFL with mouse 3T3 cell extracts), it was not possible to interchange templates between the two *in vitro* assays. Thus, the Ad2 and AdFL *in vitro* assay systems were very specific and could discriminate between a homologous and a heterologous template. Further, nuclear extracts from AdFL infected HeLa cells could initiate and partially elongate AdFL DNA-protein complex but were completely inactive when Ad2 DNA-protein complex was used as template. Thus, even in the presence of human cell proteins, Ad2 DNA cannot be replicated by mouse viral proteins.
Protein-free DNA can also serve as *in vitro* template for Ad DNA replication. The cloned terminal fragments and synthetic termini of Ad2 DNA support initiation and limited elongation *in vitro* using crude nuclear extracts from Ad2 infected HeLa cells. These protein-free termini must be longer than the first 17bp of the Ad2 genome and must be located at the end of a linearized DNA molecule. Since Ad2 and AdFL only share the first 17bp, cloned AdFL termini are inactive in the Ad2 system. The "mixed" nuclear extracts (i.e. AdFL/HeLa) retain their specificity for a mouse virus terminus and

can only use cloned AdFL termini which must be longer than 18bp at least in this *in vitro* system. Finally, nuclear extracts from AdFL infected 3T3 cells support limited DNA synthesis using cloned AdFL genome termini that are longer than 18bp. However, many nuclear preparations could also initiate, but not elongate, Ad2 origin-containing plasmids and other plasmids without Ad sequences. This demonstrates a reduced specificity of the infected mouse cell extracts with respect to template which may be due to low levels of viral- or host-coded replication factors.

Cytoplasmic extracts from Ad infected cells could efficiently support initiation of Ad2 DNA replication only when supplemented with nuclear extracts from uninfected HeLa cells. Thus, it was demonstrated that viral DNA replication is dependent on one or more host cell nuclear factors. These nuclear factors must be broadly conserved genetically since mouse 3T3 cell nuclear extracts can also complement cytoplasmic extracts from Ad2 infected HeLa cells in the Ad2 *in vitro* replication system.

6.1. ZUSAMMENFASSUNG .

In einem zellfreien Modellsystem wurde unter Verwendung von menschlichem Adenovirus Typ 2 und Mausadenovirus Typ FL die Initiation der Adenovirus DNA Replikation untersucht. Nach dem "protein-priming" Modell beginnt die DNA Replikation mit der kovalenten Bindung des biologischen Vorläufers des viralen terminalen Proteins (pTP) an den ersten Nukleotidrest des Adenovirusgenoms, dCTP. Dieser Komplex pTP-dCMP bildet den sogenannten Initiationskomplex. Mit Kernextrakten Ad2-infizierter HeLa-Zellen kann *in vitro* erfolgreich die DNA Synthese an Ad2 DNA-Protein-Komplexen initiiert werden. Eine wenn auch limitierte Elongation bis zum ersten dG-Rest ist ebenfalls möglich.
Im Verlauf der Arbeiten wurde ein *in vitro* System entwickelt, um die Initiation der Mausadenovirus FL Replikation zu untersuchen. Es konnte gezeigt werden, daß Kernextrakte aus AdFL-infizierten Maus-3T3-Zellen die Initiation und eine limitierte Elongation erlauben, wenn ein DNA-Protein-Komplex aus gereinigten AdFL Virionen verwendet wird. Der mutmaßliche Vorläufer des AdFL terminalen Proteins war mit einem Molekulargewicht von 80K kleiner als der Vorläufer des terminalen Proteins von Ad2 (87K). Die in den beiden homologen *in vitro* Systemen erzielten Ergebnisse, d.h., Ad2 DNA mit Extrakten menschlicher Zellen, bzw. AdFL mit Extrakten aus Maus-3T3-Zellen, erwiesen sich als reproduzierbar. Es war jedoch nicht möglich, im Maus-System Ad2, bzw. im HeLa-System AdFL *in vitro* zu initiieren. Dies deutet darauf hin, daß die *in vitro* Systeme für Ad2 und AdFL sehr spezifisch sind und zwischen homologen und heterologen DNA Substraten für die Initiationsreaktion unterscheiden können. Kernextrakte aus AdFL-infizierten HeLa-Zellen konnten AdFL DNA-Protein-Komplexe initiieren und teilweise elongieren, erlaubten jedoch nicht die Initiation und Elongation von Ad2 DNA-Protein-Komplexen. Diese Experimente belegen, daß Ad2 DNA selbst in Gegenwart von zellulären Faktoren aus HeLa-Zellen nicht von Mausvirusproteinen repliziert werden kann.

Proteinfreie virale DNA kann ebenfalls in vitro als Substrat für die Replikation dienen. Klonierte Endfragmente von Adenovirus DNA und synthetisch hergestellte Ad2 DNA Termini werden in vitro initiiert und teilweise elongiert, wenn Rohextrakte aus Ad2-infizierten HeLa-Zellen verwendet werden. Diese proteinfreien Termini müssen jedoch länger als die ersten 17 Basenpaare des Ad2 Genoms sein und in einem linearisierten Vektor am Ende des DNA Molekuls liegen. Da die DNA Sequenzen der Termini von Ad2 und AdFL nur in den ersten 17 Basenpaaren übereinstimmen, werden klonierte AdFL Termini im Ad2 System nicht initiiert und elongiert.

In AdFL/HeLa-Mischextrakten bleibt die Spezifität für AdFL Termini erhalten, und klonierte AdFL Termini werden - zumindest in dem in der Arbeit verwendeten in vitro System - nur initiiert und elongiert, wenn sie länger als 18 Basenpaare sind.

Kernextrakte aus AdFL-infizierten Maus-3T3-Zellen erlauben ebenfalls eine begrenzte DNA Synthese an klonierten AdFL Genomen, wenn sie länger als 18 Basenpaare sind. Mit vielen Kernextrakten konnte allerdings die Initiation, nicht aber die Elongation von Plasmiden beobachtet werden, die eine Sequenz für den Ursprung der Ad2 DNA Replikation trugen oder überhaupt keine Adenovirus DNA Sequenzen enthielten. Dies deutet auf eine reduzierte Spezifität der Extrakte aus infizierten Mauszellen bezüglich der zu verwendenden DNA Matrize hin. Die Ursache hierfür sind möglicherweise geringe Mengen von virus- oder wirtszellkodierten Replikationsfaktoren.

Zytoplasmatische Extrakte aus Adenovirus-infizierten Zellen waren nur dann in der Lage, die Ad2 DNA Replikation wirkungsvoll zu initiieren, wenn sie mit Kernextrakten uninfizierter HeLa-Zellen supplementiert wurden. Dies beweist, daß die virale DNA Replikation von einem oder mehreren Wirtsfaktoren aus dem Zellkern abhängig ist. Solche Faktoren müssen genetisch weitgehend konserviert sein, weil Kernextrakte aus Maus-3T3-Zellen in Verbindung mit zytoplasmatischen Extrakten aus Adenovirus-infizierten Zellen ebenfalls ein funktionsfähiges Ad2 in vitro Replikationssystem ergeben.

7. LITERATURE.

Akusjärvi, G. and Persson, H. (1981). Gene and mRNA for precursor polypeptide V1 from adenovirus type 2. J. Virol. 38 pp 469 - 482.

Aleström, P., Akusjärvi, G., Pettersson, M. and Pettersson, U. (1982). DNA sequence analysis of the region encoding the terminal protein and the hypothetical N-gene product of adenovirus type 2. J. Biol. Chem. 257 pp 13492 - 13498.

Antoine, G., Aleström. P., Schilling, R., Pettersson. U. and Winnacker, E.-L. (1982). Selective expression of mouse adenovirus FL in human cells. The EMBO Journal 1 pp 453 - 459.

Antoine, G. (1984). Expression von Maus-adenovirus. Inaugural-Dissertation. Inst. f. Biochem., Uni. München. FRG.

Arens, M. and Yamashita, T. (1978). In vitro termination of adenovirus DNA synthesis by a soluble replication complex. J. Virol. 25 pp698 - 702.

Ariga, H. and Shimojo, H. (1977). Initiation and termination sites of adenovirus 12 DNA replication. Virology 78 pp415 - 424.

Ariga, H. and Shimojo, H. (1978). Initiation and termination sites of adenovirus 12 DNA replication. II. Analysis of pulse-labelled oligonucleotides derived from 5'-termini in the DNA strand. Virology 85 pp98 - 108.

Arrand, J. R. and Roberts, R.J. (1979). The nucleotide sequences at the termini of Ad2 DNA. J. Mol. Biol. 128 pp577 - 594.

Bhatti, A. R. and Weber, J. (1979). Protease of adenovirus type 2. J. Biol. Chem. 254 pp12265 - 12268.

Bodnar, J. W. and Pearson, G. D. (1980a). Kinetics of adenovirus DNA replication. I. Rate of adenovirus DNA replication. Virology 100 pp208 - 211.

Bodnar, J. W. and Pearson, G.D. (1980b). Kinetics of adenovirus DNA replication. II. Initiation of adenovirus DNA replication. Virology 105 pp357 - 370.

Bolton, A.E. and Hunter, W.M. (1973). The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Biochem. J. 133 pp529 - 539.

Bourgaux, P., Delbecchi, L. and Bourgaux-Ramoisy, D. (1976). Initiation of adenovirus type 2 DNA replication. J. Virol. 72 pp89 - 98.

Brison, O., Kedinger, C. and Wilhelm, J. (1977). Enzymatic properties of viral replication complexes isolated from adenovirus type 2 infected HeLa cell nuclei. J. Virol. 24 pp423 - 435.

Brown, D.T., Westphal, M., Burlingham, B.T., Winterhoff, U. and Doerfler, W. (1975). Structure and composition of the adenovirus type 2 core. J. Virol. 16 pp366 - 387.

Bucknall, R.A., Moores, H., Simms, R. and Hesp, B. (1973). Antimicrob. Agents Chemother. 4 pp294 - 298.

Carusi, E.A. (1977). Evidence for blocked 5'-termini in human adenovirus DNA. Virology 76 pp380 - 394.

Cavalier-Smith, T. (1974). Palindromic base sequences and replication of eukaryotic chromosome ends. Nature 250 pp467 - 470.

Challberg, M.D. and Kelly, T.J.Jr. (1979a). Adenovirus DNA replication *in vitro* . Proc. Natl. Acad. Sci. USA 76 pp655 - 659.

Challberg, M.D. and Kelly, T.J.Jr. (1979b). Adenovirus DNA replication *in vitro* . Origin and direction of daughter strand synthesis. J. Mol. Biol. 135 pp999 - 1011.

Challberg, M.D., Desiderio, S.V. and Kelly, T.J.Jr. (1980). Adenovirus DNA replication *in vitro* : characterization of a protein covalently linked to nascent DNA strands. Proc. Natl. Acad. Sci. USA 77 pp5105 - 5109.

Challberg, M.D. and Kelly, T.J.Jr. (1981). Processing of the adenovirus terminal protein. J. Virol. 38 pp272 - 277.

Challberg, M.D. and Kelly, T.J.Jr. (1982). Eukaryotic DNA replication: viral and plasmid model systems. Ann. Rev. Biochem. 51 pp901 - 934.

Challberg. M.D., Ostrove, J.M. and Kelly, T.J.Jr. (1982). Initiation of adenovirus DNA replication: detection of covalent complexes between nucleotide and the 80-kilodalton terminal protein. J. Virol. 41 pp265 - 270.

Chen, T.R. (1977). In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. Exp. Cell. Res. 104 pp255 - 262.

Chinnadurai, G., Chinnadurai, S. and Green, M. (1978). Enhanced infectivity of adenovirus type 2 DNA and a DNA-protein complex. J. Virol. 26 pp195 - 199.

Clewell, D.B. (1972). Nature of ColE1 plasmid replication in Escherichia coli in the presence of chloramphenicol. J. Bacteriol. 110 pp667 - 676.

Coombs, D.H., Robinson, A.J., Bodnar, J.W., Jones, C.J. and Pearson, G.D. (1978). Detection of DNA-protein complexes: the adenovirus DNA terminal protein and HeLa DNA-protein complexes. Cold Spring Harbor Symp. Quant. Biol. 43 pp741 - 753.

Daniell, E. (1976). Genome structure of incomplete particles of adenovirus. J. Virol. 19 pp685 - 708.

De Jong, P.J., Kwant, M.M., van Driel, W., Jansz, H.S. and van der Vliet, P.C. (1983). The ATP requirements of adenovirus type 5 DNA replication and cellular DNA replication. Virology 124 pp45 - 48.

Desiderio, S.V. and Kelly, T.J.Jr. (1981). Structure of the linkage between adenovirus DNA and the 55000 molecular weight terminal protein. J. Mol. Biol. 145 pp319 - 337.

Doerfler, W. and Kleinschmidt, A.K. (1970). Denaturation pattern of the DNA of adenovirus type 2 as determined by electron microscopy. J. Mol. Biol. 50 pp579 - 593.

Dörper, T. and Winnacker, E.-L. (1983). Improvements in the phosphoramidite procedure for the synthesis of oligodeoxyribonucleotides. Nucleic Acids Res. 11 pp2575 - 2584.

Dressler, D. (1975). The recent excitement in the DNA growing point problem. Ann. Rev. Microbiol. 29 pp525 - 579.

Dulbecco, R. and Freeman, G. (1959). Plaque production by the polyoma virus. Virology 8 p396 - 397.

Dussaix, E., Faucon-Bignet, N., Samolyk, D., Boni, C. and Tournier, P. (1982). Restricted replication of mouse adenovirus strain FL in mouse Balb/c cells transformed by simian adenovirus 7. Virology 116. pp641 - 645.

Eagle, H. (1959). Amino acid metabolism in mammalian cell cultures. Science 130 pp432 - 437.

Eisenberg, S., Griffith, J. and Kornberg, A. (1977). ϕX174 cistron A protein is a multifunctional enzyme in DNA replication. Proc. Natl. Acad. Sci. USA 74 pp3198 - 3202.

Eisenberg, S. (1980). Cleavage of ϕX single-stranded DNA by gene A protein and formation of a tight protein-DNA complex. J. Virol. 35 pp409 - 413.

Enns, R.E., Challberg, M.D., Ahern, K.G., Chow, K.-C., Mathews, C.Z., Astell, C.R. and Pearson, G.D. (1983). Mutational mapping of a cloned adenovirus origin. Gene 23 pp307 - 313.

Enomoto, T., Lichy, J., Ikeda, J. and Hurwitz, J. (1981). Adenovirus DNA replication in vitro : purification of the terminal protein in a functional form. Proc. Natl. Acad. Sci. USA 78 pp6779 - 6783.

Estes, M.K. (1978). Characterization of DNA-protein complexes from simian adenovirus SA7. J. Virol. 25 pp917 - 922.

Flint, S.J. (1981). Structure and genomic organization of adenoviruses. In DNA Tumour Viruses. J. Tooze (ed.) Second edition, Cold Spring Harbor Laboratory. pp383 - 442.

Fütterer, J. (1982). DNA-Protein-Komplexe in Adenovirus-infizierten Zellen. Inaugural-Dissertation, Inst. f. Biochem., Uni. München. FRG.

Fütterer, J. and Winnacker, E.-L. (1984). Adenovirus DNA replication. In The Molecular Biology of Adenoviruses. Doerfler, W. (ed.) Current Topics in Microbiology and Immunology. Springer Verlag. Berlin.

Furth, M.E. and Wickner, S.H. (1983). Lambda DNA replication. In Lambda II. Hendrix, R., Roberts, J., Stahl, F. and Weisberg, R. (eds.). Cold Spring Harbor Monograph Series, USA.

Galos, R.S., Williams, J., Binger, M.H. and Flint, J.S. (1979). Location of additional early gene sequences in the adenovirus chromosome. Cell 17 pp945 - 956.

Garon, C.F., Berry, K.W. and Rose, J.A. (1972). A unique form of terminal redundancy in adenovirus DNA molecules. Proc. Natl. Acad. Sci. USA 69 pp2391 - 2395.

Gerlich, W.H. and Robinson, W.S. (1980). Hepatitis B virus contains protein attached to the 5' terminus of its complete DNA strand. Cell 21 pp801 - 809.

Gingeras, T.R., Sciaky, D., Gelinas, R.E., Bing-Dong, J., Yen, C.E., Kelly, M.M., Bullock, P.A., Parsons, B.L., O'Neil, K.E. and Roberts, R.J. (1982). Nucleotide sequences from the adenovirus-2 genome. J. Biol. Chem. 257 pp13475 - 13491.

Girard, M., Bouche, J.-P., Marty, L., Revet, B. and Berthelot, N. (1977). Circular adenovirus DNA-protein complexes from infected HeLa cell nuclei. Virology 83 pp34 - 35.

Goding, C.R. and Russell, W.C. (1983). Adenovirus cores can function as templates in *in vitro* DNA replication. The EMBO Journal 2 pp339 - 344.

Graham, F.L., Abrahams, P.J., Mulder, C., Heijneker, H.L., Warnaar, S.O., de Vries, F.A.J., Fiers, W. and van der Eb, A.J. (1974). Studies on *in vitro* transformation by DNA and DNA fragments of human adenovirus and SV40. Cold Spring Harbor Symp. Quant. Biol. 39 pp637 - 650.

Green, M., Pina, M., Kimes, R., Wensink, P.C., MacHattie, L.A. and Thomas, C.A.Jr. (1967). Adenovirus DNA. I. Molecular weight and conformation. Proc. Natl. Acad. Sci. USA 57 pp1302 - 1309.

Green, M., Arens, M.Q., Yamashita, T., Wold, W.S.M. and Brackmann, K.H. (1978). Adenovirus 2 DNA: in vitro replication and analysis of viral early proteins. Cold Spring Harbor Symp. Quant. Biol. 43 pp755 - 767.

Green, M., Brackmann, K., Wold, W.S.M., Cartas, M., Thornton, H. and Elder, J.E. (1979a). Conserved primary sequences of the DNA terminal proteins of five different human adenovirus groups. Proc. Natl. Acad. Sci. USA 76 pp4380 - 4384.

Green, M., Mackey, J.K., Wold, W.S.M. and Rigden, P. (1979b). Thirty-one human adenovirus serotypes (Ad1 - Ad31) form five groups (A - E) based upon DNA genome homologies. Virology 93 pp481 - 492.

Green, M., Symington, J., Brackmann, K.H., Cartas, M.A., Thornton, H. and Young, L. (1981). Immunological and chemical identification of intracellular forms of adenovirus type 2 terminal protein. J. Virol. 40 p541 - 550.

Harding, N., Ito, J. and David, G.S. (1978). Identification of the protein firmly bound to the ends of bacteriophage ϕ29 DNA. Virology 84 pp279 - 292.

Hartley, J.W. and Rowe, W.P. (1960). A new mouse virus apparently related to the adenovirus group. Virology 11 pp645 - 647.

Heidekamp, F., Langevelt, S.A., Baas, P.D. and Jansz, J.S. (1980). Comparison of ϕX174, G4 and St-1 phage for site of action of A protein. Nucleic Acids Res. 8 pp2009 - 2021.

Hilleman, M.R. and Werner, J.H. (1954). Recovery of a new agent from patients with acute respiratory illness. Proc. Soc. Exp. Biol. Med. 85 pp183 - 188.

Horwitz, M.S. (1976). Bidirectional replication of adenovirus type 2 DNA. J. Virol. 18 pp307 - 315.

Huberman, J.A. (1981). New views of the biochemistry of eucaryotic DNA replication revealed by aphidicolin, an unusual inhibitor of DNA polymerase α. Cell 23 pp647 - 648.

Humphreys, G.O., Willshaw, G.A. and Anderson, E.S. (1975). A simple method for the preparation of large quantities of pure plasmid DNA. Biochim. Biophys. Acta 383 pp457 - 463.

Ikeda, J.E., Longiaru, M., Horwitz, M.S. and Hurwitz, J. (1980). Elongation of primed DNA templates by eukaryotic DNA polymerases. Proc. Natl. Acad. Sci. USA 77 pp5827 - 5831.

Ikeda, J.E., Enomoto, T. and Hurwitz, J. (1982). Adenoviral protein-primed initiation of DNA chains in vitro . Proc. Natl. Acad. Sci. USA 79 pp2442 - 2446.

Ikegami, S., Taguchi, T., Ohashi, M., Oguro, M., Nagano, H. and Mano, Y. (1978). Aphidicolin prevents mitotic cell division by interfering with the activity of DNA polymerase α. Nature 275 pp458 - 460.

Ito, J. (1978). Bacteriophage ϕ29 terminal protein: its association with the 5'-termini of the ϕ29 genome. J. Virol. 28 pp895 - 904.

Kaplan, L.M., Kleinmann, R.E. and Horwitz, M.S. (1977). Replication of adenovirus type 2 DNA in vitro . Proc. Natl. Acad. Sci. USA 74 pp4425 - 4429.

Kaplan, L.M., Ariga, H., Hurwitz, J. and Horwitz, M.S. (1979). Complementation of the temperature-sensitive defect in H5ts125 adenovirus DNA replication in vitro . Proc. Natl. Acad. Sci. USA 76 pp5534 - 5538.

Kathmann, H.-P. (1974). Isolation von temperatursensitiven Mutanten von Adenovirus Typ 2. Diplomarbeit, Inst. f. Genetik d. Uni. zu Köln.

Kelly, T.J.Jr. and Lechner, R.L. (1978). The structure of replicating adenovirus DNA molecules: characterization of DNA-protein complexes from infected cells. Cold Spring Harbor Symp. Quant. Biol. 43 pp721 - 728.

Kitamura, N., Semler, B.L., Rothberg, P.G., Larsen, G.R., Adler, C.J., Dorner, A.J., Emini, E.A., Hanecak, R., Lee, J.J., van der Werf, S., Anderson, C.W. and Wimmer, E. (1981). Primary structure, gene organization and polypeptide expression of poliovirus RNA. Nature 291 pp547 - 553.

Klessig, D.F. and Chow, L.T. (1980). Incomplete splicing and deficient accumulation of the fiber messenger RNA in monkey cells infected by human adenovirus type 2. J. Mol. Biol. 139 pp221 - 242.

Kornberg, A. (1980). DNA replication. W.H. Freeman and Company, USA.

Kwant, M.M. and van der Vliet, P.C. (1980). Differential effect of aphidicolin on adenovirus DNA synthesis and cellular DNA synthesis. Nucleic Acids Res. 8 pp3993 - 4007.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 pp680 - 685.

Larsen, S.H. and Nathans, D. (1977). Mouse adenovirus: growth of plaque-purified FL virus in cell lines and characterization of viral DNA. Virology 82 pp182 - 195.

Larsen, S.H., Margolskee, R.F. and Nathans, D. (1979). Alignment of the restriction map of mouse adenovirus FL with that of human adenovirus 2. Virology 97 pp406 - 414.

Lechner, R.L. and Kelly, T.J.Jr. (1977). The structure of replicating adenovirus 2 DNA molecules. Cell 12 pp1007 - 1020.

Lee, Y.F., Nomoto, A., Detjen, B.M. and Wimmer, E. (1977). A protein covalently linked to poliovirus genome RNA. Proc. Natl. Acad. Sci. USA 74 pp59 - 63.

Lichy, J.H., Horwitz, M.S. and Hurwitz, J. (1981). Formation of a covalent complex between the 80000-dalton adenovirus terminal protein and 5'-dCMP in vitro . Proc. Natl. Acad. Sci. USA 78 pp2678 - 2682.

Lichy, J.H., Field, J., Horwitz, M.S. and Hurwitz, J. (1982). Separation of the adenovirus terminal protein precursor from its associated DNA polymerase: role of both proteins in the initiation of adenovirus DNA replication. Proc. Natl. Acad. Sci. USA 79 pp5225 - 5229.

Longiaru, M., Ikeda, J., Jarkovsky, Z., Horwitz, S.B. and Horwitz, M.S. (1979). The effect of aphidicolin on adenovirus DNA synthesis. Nucleic Acids Res. 6 pp3369 - 3386.

Longiaru, M. and Horwitz, M.S. (1981). Chinese hamster ovary cells (CHO) replicate adenovirus DNA. Mol. Cell. Biol. 1 pp208 - 215.

Maizel, J.V.Jr., White, D.O. and Scharff, M.D. (1968). The polypeptides of adenovirus. II. Soluble proteins, cores, top components and the structure of the virion. Virology 36 pp126 - 136.

Mellado, R.P., Penalva, M.A., Inciarte, M.R. and Salas, M. (1980). The protein covalently linked to the 5'termini of the DNA of Bacillus subtilis phage Ø29 is involved in the initiation of DNA replication. Virology 104 pp84 - 96.

Mirza, M.A.A. and Weber, J. (1977). Genetic analysis of adenovirus type 2. VII. Cleavage modified affinity for DNA of internal virion proteins. Virology 80 pp83 - 97.

Nagata, K., Guggenheimer, R.A., Enomoto, T., Lichy, J.H. and Hurwitz, J. (1982). Adenovirus DNA replication in vitro : identification of a host factor that stimulates synthesis of the preterminal protein-dCMP complex. Proc. Natl. Acad. Sci. USA 79 pp6438 - 6442.

Nagata, K., Guggenheimer, R.A. and Hurwitz,J. (1983a). Adenovirus DNA replication in vitro : synthesis of full-length DNA with purified proteins. Proc. Natl. Acad. Sci. USA 80 pp4266 - 4270.

Nagata, K., Guggenheimer, R.A. and Hurwitz, J. (1983b). Specific binding of a cellular DNA replication protein to the origin of replication of adenovirus DNA. Proc. Natl. Acad. Sci. USA 80 pp6177 - 6181.

Nomoto, A., Detjen, B., Pozatti, R. and Wimmer, E. (1977). The location of the polio genome protein in viral RNAs and its implication for RNA synthesis. Nature 268 pp208 - 213.

Padmanabhan, R. and Padmanabhan, R.V. (1977). Specific interactions of a protein(s) at or near the termini of adenovirus 2 DNA. Biochem. Biophys. Res. Commun. 75 pp955 - 964.

Pearson, G.D., Chow, K.-C., Enns, R.E., Ahern, K.G., Corden, J.L. and Harpst, J.A. (1983). In vitro replication directed by a cloned adenovirus origin. Gene 23 pp293 - 305.

Pedrali-Noy, G. and Spadari, S. (1980). Mechanism of inhibition of Herpes simplex virus and Vaccinia virus DNA polymerases by aphidicolin, a highly specific inhibitor of DNA replication in eucaryotes. J. Virol. 36 pp457 - 464.

Penalva, M.A. and Salas, M. (1982). Initiation of phage ϕ29 DNA replication in vitro : Formation of a covalent complex between the terminal protein, p3, and 5'-dAMP. Proc. Natl. Acad. Sci. USA 79 pp5522 - 5526.

Philipson, L. and Lindberg, U. (1974). Reproduction of adenoviruses. In Comprehensive Virology, H. Fraenkel-Conrat and R.R. Wagner (eds.). 3 Plenum Press, New York, pp143 - 227.

Philipson, L. (1983). Structure and assembly of adenoviruses. In The Molecular Biology of Adenoviruses 1. Doerfler, W. (ed.). Current Topics in Microbiology and Immunology. 109 Springer Verlag, Berlin.

Pincus, S., Robertson, W. and Rekosh, D. (1981). Characterization of the effect of aphidicolin on adenovirus DNA replication: evidence in support of a protein primer model of initiation. Nucleic Acids Res. 9 pp4919 - 4938.

Rabson, A.S., O'Connor, G.T., Berezesky, I.K. and Paul, F.J. (1964). Enhancement of adenovirus growth in African green monkey kidney cell cultures by SV40. Proc. Soc. Exp. Biol. Med. 116 pp187 - 190.

Reiter, T., Fütterer, J., Weingärtner, B. and Winnacker, E.-L. (1980). Initiation of adenovirus DNA replication. J. Virol. 35 pp662 - 671.

Rekosh, D.M.K., Russell, W.C., Bellett, A.J.D. and Robinson, A.J. (1977). Identification of a protein linked to the ends of adenovirus DNA. Cell 11 pp283 - 295.

Rekosh, D. (1981). Analysis of the DNA-terminal protein from different serotypes of human adenovirus. J. Virol. 40 pp329 - 333.

Revet, B. and Benichou, D. (1981). Electron microscopy of Ad5 replicating molecules after *in vivo* photocrosslinking with trioxsalen. Virology 114 pp60 - 70.

Revie, D., Tseng, B.Y., Grafstrom, R.H. and Goulian, M. (1979). Covalent association of a protein with replicative form DNA of parvovirus H-1. Proc. Natl. Acad. Sci. USA 76 pp5539 - 5543.

Robinson, A.J., Younghusband, H.B. and Bellett, A.J.D. (1973). A circular DNA-protein complex from adenoviruses. Virology 56 pp54 - 69.

Robinson, A.J. and Bellett, A.J.D. (1974). A circular DNA-protein complex from adenoviruses and its possible role in DNA replication. Cold Spring Harbor Symp. Quant. Biol. 39 pp523 - 531.

Rowe, W.P., Huebner, R.J., Gillmore, L.K., Parrott, R.H. and Ward, T.G. (1953). Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proc. Soc. Exp. Biol. Med. 84 pp570 - 573.

Ruben, M., Bacchetti, S. and Graham, F. (1983). Covalently closed circles of adenovirus 5 DNA. Nature 301 pp172 - 174.

Salas, M., Mellado, R.P., Vinuela, E. and Sogo, J.M. (1978). Characterization of a protein covalently linked to the 5'-termini of the DNA of Bacillus subtilis phage ϕ29. J. Mol. Biol. 119 pp269 - 291.

Schilling, R., Weingärtner, B. and Winnacker, E.-L. (1975). Adenovirus type 2 DNA replication. II. Termini of DNA replication. J. Virol. 16 pp767 - 774.

Seif, I., Khoury, G. and Dhar, R. (1979). The genome of human papovavirus BKV. Cell 18 pp963 - 977.

Sharp, P.A., Sugden, B. and Sambrook, J. (1973). Detection of two restriction endonuclease activities in Haemophilus parainfluenza using analytical agarose-ethidium bromide electrophoresis. Biochemistry 12 pp3055 - 3063.

Sharp, P.A., Moore, C. and Haverty, J.L. (1976). The infectivity of adenovirus 5 DNA-protein complex. Virology 75 pp442 - 458.

Sharp, P.A. (1977). Adenovirus strand nomenclature: a proposal. J. Virol. 22 pp830 - 831.

Shinagawa, M. and Padmanabhan, R. (1979). Nucleotide sequence at the inverted terminal repetition of adenovirus type 2 DNA. Biochem. Biophys. Res. Commun. 87 pp671 - 678.

Smart, J.E. and Stillman, B.W. (1982). Adenovirus terminal protein precursor. J. Biol. Chem. 257 pp13499 - 13506.

Soberon, X., Covarrubias, L. and Bolivar, F. (1980). Construction and characterization of new cloning vehicles. IV. Deletion derivatives of pBR322 and pBR325. Gene 9 pp287 - 305.

Stillman, B.W., Bellett, A.J.D. and Robinson, A.J. (1977). Replication of linear adenovirus DNA is not hairpin-primed. Nature 269 pp723 - 725.

Stillman, B.W. and Bellet, A.J.D. (1978). Replication of DNA in adenovirus infected cells. Cold Spring Harbor Symp. Quant. Biol. 43 pp729 - 739.

Stillman, B.W. and Bellett, A.J.D. (1979). An adenovirus protein associated with the ends of replicating DNA molecules. Virology 93 pp69 - 79.

Stillman, B.W. (1981). Adenovirus DNA replication *in vitro* : a protein linked to the 5'-end of nascent DNA strands. J. Virol. 37 pp139 - 147.

Stillman, B.W., Lewis, J.B., Chow, L.T., Mathews, M.B. and Smart, J.E. (1981). Identification of the gene and mRNA for the adenovirus terminal protein precursor. Cell 23 pp497 - 508.

Stillman, B.W., Topp, W.C. and Engler, J.A. (1982). Conserved sequences at the origin of adenovirus DNA replication. J. Virol. 44 pp530 - 537.

Stillman, B.W., Tamanoi, F. and Mathews, M.B. (1982b). Purification of an adenovirus-coded DNA polymerase that is required for initiation of DNA replication. Cell 31 pp613 - 623.

Stillman, B.W. (1983). The replication of adenovirus DNA with purified proteins. Cell 35 pp7 - 9.

Sussenbach, J.S. and van der Vliet, P.C. (1972). Viral DNA synthesis in isolated nuclei from adenovirus infected KB cells. FEBS Lett. 21 pp7 - 15.

Sussenbach, J.S. and van der Vliet, P.C. (1973). Studies on the mechanism of replication of adenovirus DNA. I. The effect of hydroxyurea. Virology 54 pp299 - 303.

Sussenbach, J.S. and Kuijk, M.G. (1977). Studies on the mechanism of replication of adenovirus DNA. V. The location of termini of replication. Virology 77 pp149 - 157.

Sussenbach, J.S. and Kuijk, M.G. (1978a). The mechanism of replication of adenovirus DNA. VI. Localization of the origins of displacement synthesis. Virology 84 pp509 - 517.

Sussenbach, J.S. and Kuijk, M.G. (1978b). Initiation of adenovirus DNA replication does not occur via a hairpin mechanism. Nucleic Acids Res. 5 pp1289 - 1295.

Tamanoi, F. and Stillman, B.W. (1982). Function of adenovirus terminal protein in the initiation of DNA replication. Proc. Natl. Acad. Sci. USA 79 pp2221 - 2225.

Tamanoi, F. and Stillman, B.W. (1983). Initiation of adenovirus DNA replication *in vitro* requires a specific DNA sequence. Proc. Natl. Acad. Sci. USA 80 pp6446 - 6450.

Temple, M. P. (1980). Characterization of mouse adenovirus and other prerequisite experiments to the definition of the adenovirus replicon. Inaugural-Dissertation, Inst. f. Biochem., Uni. München. FRG.

Temple, M., Antoine, G., Delius, H., Stahl, S. and Winnacker, E.-L. (1981). Replication of mouse adenovirus strain FL DNA. Virology 109 pp1 - 12.

Tomizawa, J. and Ogawa, T. (1968). Replication of phage lambda DNA. Cold Spring Harbor Symp. Quant. Biol. 33 pp533 - 551.

Tolun, A. and Pettersson, U. (1975). Termination sites for adenovirus type 2 DNA replication. J. Virol. 16 pp759 - 766.

Tolun, A., Aleström, P. and Pettersson, U. (1979). Sequence of inverted terminal repetitions from different adenoviruses: demonstration of conserved sequences and homology between SA7 termini and SV40 DNA. Cell 17 pp705 - 713.

van Bergen, B.G.M., van der Ley, P.A., van Driel, W., van Mansfield, A.D.M. and van der Vliet, P.C. (1983). Replication of origin containing adenovirus DNA fragments that do not carry the terminal protein. Nucleic Acids Res. 11 pp1975 - 1989.

van Wielink, P.S., Naaktgeboren, N. and Sussenbach, J.S. (1979). Presence of protein at the termini of intracellular adenovirus type 5 DNA. Biochem. Biophys. Acta 563 pp89 - 99.

Vieira, J. and Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19 pp259 - 268.

Vogt, P.K. (1969). Immunofluorescent detection of viral antigens. In Fundamental Techniques in Virology. K. Habel and N.P. Salzman (eds.). Academic Press, NY/London. pp316 - 326.

Watson, J.D. (1972). Origin of concatemeric T7 DNA. Nature New Biol. 239 pp197 - 201.

Weber, J. (1976). Genetic analysis of adenovirus type 2. III. Temperature sensitivity of processing of viral proteins. J. Virol. 17 pp462 - 471.

Weingärtner, B., Winnacker, E.-L., Tolun, A. and Pettersson, U. (1976). Two complementary strand-specific termination sites for adenovirus DNA replication. Cell 9 pp259 - 268.

Weissbach, A. (1975). Vertebrate DNA polymerases. Cell 5 pp101 - 108.

Weissbach, A. (1977). Eukaryotic DNA polymerases. Ann. Rev. Biochem. 46 pp25 - 47.

Wigand, R., Gelderblom, H. and Ozel, M. (1977). Biological and biophysical characteristics of mouse adenovirus strain FL. Arch. Virol. 54 pp131 - 142.

Williams, J.F. (1970). Enhancement of adenovirus plaque formation on HeLa cells by magnesium chloride. J. Gen. Virol. 9 pp251 - 255.

Winnacker, E.-L. (1975). Adenovirus type 2 DNA replication. I. Evidence for discontinuous DNA synthesis. J. Virol. 15 pp744 - 758.

Winnacker, E.-L. (1978). Adenovirus DNA: Structure and function of a novel replicon. Cell 14 pp761 - 773.

Wolfson, J. and Dressler, D. (1972). Adenovirus-2 DNA contains an inverted terminal repetition. Proc. Natl. Acad. Sci. USA 69 pp3054 - 3057.

Yamaguchi, K., Shiroki, K. and Shimojo, H. (1977). The relationship between the formation of inclusions and viral DNA synthesis in adenovirus 12 infected cells. Virology 80 pp136 - 148.

Yamashita, T., Arens, M. and Green, M. (1975). Adenovirus deoxyribonucleic acid replication. II. Synthesis of viral deoxyribonucleic acid *in vitro* by a nuclear membrane fraction from infected KB cells. J. Biol. Chem. 250 pp3273 - 3279.

Yehle, C.O. (1978). Genome linked protein associated with the 5'-termini of bacteriophage ϕ29 DNA. J. Virol. 27 pp776 - 783.

Young, C.W. and Hodas, S. (1964). Hydroxyurea: inhibitory effect on DNA metabolism. Science 146 pp1172 - 1174.

Zain, B.S. and Roberts, R.J. (1977). A new specific endonuclease from Xanthomonas badrii. J. Mol. Biol. 115 pp249 - 255.

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